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PRINCIPAL INVESTIGATOR: Rafael A. Fridman, Ph.D.

CONTRACTING ORGANIZATION: Wayne State University
Detroit, Michigan 48202

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Detroit, Michigan 48202

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Metastasis is the major cause of death in breast cancer patients. Metastasis formation depends on the ability of the tumor cells to degrade extracellular matrices (ECM) using specialized proteinases including matrix metalloproteinases (MMPs) and cathepsins. We have studied the role of tumor-stroma interactions and extracellular matrix in the expression, regulation and cell surface localization of MMP-2, MMP-9 and MT1-MMP, three members of the MMP family, and cathepsins in breast epithelial and fibroblast cells. We report on the association of latent MMP-9 to the surface of breast epithelial cells and show that the pro-α2(IV) chain of collagen IV specifically mediates binding of the enzyme to the cell surface. The importance of the stroma in regulation of MMP and cathepsins expression was addressed using fibroblasts isolated from breast tumors cultured in collagen I gels. These studies demonstrated that culture of breast fibroblasts on collagen I induces proMMP-2 activation. Here we report some of the biochemical and molecular mechanisms involved in the collagen I-induced proMMP-2 activation and the role of MT1-MMP and TIMP-2 in this process. We have also examined the expression of cathepsins B and D in breast fibroblasts grown on collagen I.

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1. Toth, M., Gervasi, D.C., and Fridman, R. (1997) Phorbol ester-induced cell surface association of matrix metalloproteinase-9 in human MCF10A breast epithelial cells, Cancer Res, in press.

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5. INTRODUCTION

Tumor metastasis is the major cause of treatment failure in breast cancer patients. Numerous studies have shown that metastasis formation depends on the ability of the tumor cells to invade basement membranes and connective tissue matrices in a process involving a specialized group of enzymes capable of degrading extracellular matrix (ECM) components (1-3). Studies with various enzyme systems including the serine proteases (4), the matrix metalloproteinases (MMPs) (5-11) and the cathepsins (12) have shown that the degradation of ECM during tumor cell invasion is the result of a collaborative action between enzymes, enzyme receptors and enzyme inhibitors produced by both the tumor and the stroma cells. For example, tumor cells may utilize stromal enzymes during their invasion of ECM by binding enzymes to specific cell surface receptors (13) and/or by producing enzymes capable of activating stromal proteases (14). In turn, stromal cells can induce the expression of ECM-degradative enzymes in tumor cells (15) or they can produce specific enzyme inhibitors in response to the degradative activity produced by tumor cells (16).

Matrix Metalloproteinases. The MMPs are a family of highly conserved zinc-dependent proteinases capable of degrading many extracellular matrix (ECM) components (17). Although all the MMPs can degrade ECM, several of them, in particular, were shown to be associated with breast cancer. These include MMP-2 (gelatinase A, 72 kDa type IV collagenase), MMP-9 (gelatinase B, 92 kDa type IV collagenase), stromelysin-3, matrylisin, collagenase-3 and membrane type-MMP (MT-MMP), in particular MT1-MMP.The MMPs, except stromelysin-3 and the MT-MMPs, are secreted in a latent form that requires activation to become proteolytically active (17). In the case of stromelysin-3, activation occurs intracellularly and it is mediated by a furin-like enzyme (18). The mechanism of MT-MMP activation is still unknown but may also involve intracellular activation mediated by furin (22). Proenzyme activation is a critical event in regulation of MMP activity and may be essential for ECM degradation during tumor cell invasion. The physiological mechanisms responsible for MMP activation in breast cancer are not completely understood, but may involve the action of other proteases, including other MMPs. Previous studies have shown that MT1-MMP, an MMP present in the plasma membrane, may be the physiological activator of MMP-2 (19-22). The plasma membrane-dependent activation of MMP-2 is induced in cultured cells by treatment with phorbol ester (TPA) (20,21), concanavalin A (19,20,23,24), transforming growth factor-ß (20), or collagen type I (25). However, the mechanism involved in the induction of activation of MT1-MMP *in vivo* is unknown. Since activation of MMP-2 has been shown to be induced by a collagen I gel and since fibroblasts are the major producers of MT1-MMP in breast tumor (38), it is likely that the interactions of the stromal fibroblasts with collagen I may play an important role in the physiological activation of MMP-2 by MT1-MMP in breast tumors.

Tissue Inhibitors of Metalloproteinases. The MMPs are all inhibited by the tissue inhibitor of metalloproteinases (TIMPs), a conserved family of low molecular weight proteins that presently includes TIMP-1 (30 kDa) (26), TIMP-2 (21 kDa) (27,28), TIMP-3 (22 kDa) (29) and TIMP-4 (39). TIMP-1 and TIMP-2 have been shown to inhibit MMPs by forming a stoichiometric complex with the active species (1,17). The association of TIMP-1 and TIMP-2 with the gelatinases, however, is unique since the inhibitors can also form a stable complex with the latent enzymes. For instance, TIMP-1 is capable of binding to the latent form of MMP-9 (30) whereas TIMP-2 can form a complex with MMP-2 (27). This unique interaction of the TIMPs with the proenzymes may provide an additional level of regulation by preventing generation of full enzymatic activity (31,32). However, recent studies have shown a possible role of TIMP-2 on the activation of MMP-2 by MT1-MMP on the cell surface (33).

Epithelial-Stromal Regulation of ECM-degrading Proteases in Breast Cancer. In breast cancer, expression of MMPs, in particular of the gelatinases (8,34-36) and stromelysin-3 (5), have been suggested to play a role in tumor progression. Immunohistochemical studies showed elevated expression of MMP-2 and MMP-9 in the tumor cells (34,35). *In situ* hybridization studies, however, showed mRNA expression for enzymes and TIMP-2 in the fibroblasts around invasive tumor cell clusters (8) suggesting an important role for the stromal cells in MMP expression in breast cancer. In the case of the of MMP-2, it was speculated that the activator of this enzyme, the recently identified MT1-MMP (22), was probably localized in the breast cancer cells. This would allow activation of the stromal MMP-2 enzyme on the surface of the tumor cells. However, in a recent study the mRNA for MT-MMP was also localized in the tumor stroma of breast cancer tissues (38). Interestingly, it has been recently shown that the MT1-MMP protein is mainly found in the breast cancer cells (40). The reason for this differential localization is unknown but may suggest a differential stability of MT1-MMP protein and mRNA in epithelial and stroma cells or alternatively the interesting possibility that MT1-MMP may be released from the fibroblasts membranes and bind to the surface of the breast cancer cells. Recent studies demonstrated that culture of cells on collagen I induces proMMP-2 activation. Since tumor fibroblasts are surrounded by a dense matrix of collagen I, these studies suggested an important role of stroma-matrix interactions in the generation of active MMPs. Upon activation on the fibroblast surface, MMP-2 may be released and subsequently may bind to the surface of breast cancer cells where it can be localized (40). In the case of MMP-9, it has also been shown that the protein is expressed on the surface of the breast cancer cells (41). However, like MMP-2 and MT1-MMP, the mRNA of MMP-9 is localized in the stroma in particular in fibroblasts, macrophages and endothelial cells (42). This raises the question as how MMP-9 associates with the breast epithelial cells in breast tumors. MMP-9 does not bind to MT1-MMP on the cell surface and no other specific receptor/activator for MMP-9 has been reported in breast cancer cells. Therefore, the mechanisms of cell surface association of MMP-9 remain unknown. Nevertheless, the association of MMP-2 and MMP-9 protein with the surface of breast cancer cells suggest an important role for tumor-stroma interactions in the regulation of MMPs in breast cancer.

6. BODY OF REPORT

Response to the Reviewer's Critiques to our Previous Report (95-96)

We thank the reviewers for their critiques. Following are our responses.

Contractual Issues.

"While results have been presented for the determination of the expression and localization of cathepsin B and D in the MCF-10 variants, no data were provided. Similarly, no data have been provided for Tasks 1 and 2 of years 2-3 despite the presentation of results".

Regarding, cathepsin B and D expression, these data have been now included.

Technical Issues.

"It is recommended that the PI provide supporting data in the form of tables and/or graphs in all future Annual and Final reports."

We have attempted in this report to follow this recommendation when possible. However, in the course of our studies, we obtained many negative and disappointing results. These negative data is usually explained by text only.

6.1 EXPERIMENTAL METHODS

During the 1996-1997 period we have concentrated in two major aspects. One, are the studies aimed at understanding the surface localization of MMP-9 in breast epithelial cells. Although these studies were not initially planned in the original application, the findings with MMP-9 on the surface of breast epithelial cells and the characterization of the binding mechanism, reveal a critical aspect in breast cancer invasion. Namely, the ability of the breast cancer cells to use proteases produced by stromal cells for their invasive properties. Second, we have investigated in detail the mechanism of collagen I-induced activation of proMMP-2 in fibroblasts isolated from breast tumors. Some of these studies have been published (please, see **Appendix**) and others are now being ready for submission.

NOTE: In the present 96-97 report, we have only included methods that were not described in our previous reports or in the original application. Furthermore, some of the methods are described in our recent publications that are attached as Appendix to save space. Therefore, we respectfully ask the reviewer to relate to these publications for information regarding specific methodology.

6.1.a Specific Methods To Study the Activation of proMMP-2 in Primary Breast Fibroblasts.

Antibodies. A rabbit polyclonal antibody raised against a synthetic peptide (RFNEELRAVDSEYPNIK) derived from the amino acid sequence of human MT1-MMP was produced by and purchased from Genetics Research, Inc. (Huntsville, AL). Antibodies to MMP-2, TIMP-2 were reported previously (41).

Cell Culture. The isolation and characterization of the primary breast fibroblasts were described in last year's report. The cells were cultured in Waymouth's MB752-1 media supplemented with 15% fetal bovine serum (FBS).

Rat Collagen I Isolation. Collagen type I was extracted from rat tails using 01% acetic acid by stirring 1 g of tendon in 300 ml acetic acid (0.1%) for 48 hrs. Then, the solution was centrifuged to remove debris and the sup containing the collagen I was stored at 4°C. The purity of the collagen I prep was determined by SDS-PAGE and silver staining.

Preparation of Collagen I Gels. Six ml of cold collagen I solution in the acetic acid were mixed in ice with 1 ml of stock media (stock media: 20 ml 10X MEM, 20 ml FBS, 10 ml 7.5% Na_2HCO_3, 2 ml 100X penicillin-streptomycin), 0.30 ml 7.5% Na_2HCO_3 and 0.25 ml 0.34 N NaOH. Then, 1 ml from the mixture was added to a 60 mm dish for a final concentration of 0.632 mg collagen/dish. The collagen I solution was then allowed to polymerize for 10 min at room temperature followed by 30 min incubation at 37°C. The gels were then covered with serum-free MEM until used.

Pulse Chase Analyses. Breast fibroblasts were grown to 80% confluence in 60 mm dishes coated or not with collagen I or treated or not with ConA (20 μg/ml).The media was aspirated and the cell monolayer was washed twice with warm (37°C) PBS followed by incubation (45 min) with 1 ml/dish of starving media (DMEM without methionine supplemented with 25 mM Hepes). The cells were then pulsed with 500 μCi/ml of [^{35}S]methionine in starving media (0.6 ml/dish) for 15 min at 37°C. After the pulse, the dishes were placed on ice, the medium was aspirated and the cells were washed twice gently with PBS before addition of 1 ml/dish of chase media (DMEM with 10% fetal bovine serum and 4.8 mM methionine). At the end of the chase period (0-120 min at 37°C), the media was collected, centrifuged (5 min, 12000 x g) and transferred to a clean tube with the addition of harvest buffer (60 mM Tris-HCl pH 7.5 containing 0.5% SDS, 2 mM EDTA and 10 mM methionine, final concentrations). The samples were boiled (3 min), centrifuged and transferred to a new tube with the addition of 5 mM iodoacetamide, 2.5 % Triton X-100 and 20 μg/ml aprotinin (final concentrations). The cell monolayers were washed twice with D-PBS and lysed in 0.5 ml/dish of warm harvest buffer. The lysates were then subjected to five cycles of boiling and freezing followed by a brief centrifugation. The supernatants were collected into new tubes with the addition of 5 mM iodoacetamide, 2.5 % Triton X-100 and 20 μg/ml aprotinin (final concentrations). Before immunoprecipitations, the radioactivity in each sample was determined by TCA precipitation and equal number of counts were immunoprecipitated for each treatment at the same chase period. For immunoprecipitations, the media and lysate samples were incubated (16 h, 4°C) with the appropriate antibodies followed by addition of 30 μl Protein G-Sepharose beads for an additional 3-h incubation at 4°C. After recovering the beads by a brief centrifugation, the supernatant was subjected to sequential rounds of immunoprecipitations with antibodies to MT1-MMP, MMP-2 and TIMP-2. The recovered beads were washed (5 times) with cold 50 mM Tris-HCl pH 7.5 containing 150 mM NaCl, 0.1% NP-40 and 10% glycerol and resuspended in 15 μl Laemmli sample buffer with dithiotreitol followed by boiling (5 min). Samples of media and lysates were resolved by 8-16% SDS-polyacrylamide gels. Detection of radiolabeled proteins was performed by autoradiography.

Northern Blot Analyses. Total RNA was extracted from breast fibroblasts cultured or not on collagen I using the RNeasy Total RNA kit (Qiagen, Chatsworth, CA). Five μg RNA from each sample were fractionated on a 1% agarose gel in the presence of formaldehyde and then transferred onto a nylon membrane (Hybond-N, Amersham, Aylesbury, UK) followed by fixation to the membrane using an optimized UV crosslinking procedure. The blot was prehybridized at 42°C for 3 hr followed by hybridization at 42°C for 18 hr with a ^{32}P-labeled human cDNA probe for either MT1-MMP, MMP-2, TIMP-2 or cathepsin B. Then, the blots were washed twice (15 min. each) with 0.1X SSC-0.1% SDS at 65°C and autoradiographed at -80°C. RNA loading was normalized using the signal obtained with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. Quantitation of signals was performed using an Ambis™ Radioanalytic Imaging System.

6.1.b Specific Methods for the Analysis of the Cell Surface Binding of proMMP-9 to Breast Epithelial MCF10A Cells.

Cell Culture. Human immortalized MCF10A breast epithelial cells, originally isolated from a patient with fibrocystic disease of the breast. Cells were grown in Dulbecco's modified Eagle Medium (DMEM) DMEM/F-12 (1:1) (Gibco, Grand Island, NY) medium supplemented with 5% horse serum, insulin (10 μg/ml), penicillin (100 IU/ml), streptomycin (100 μg/ml), Fungizone (0.25 μg/ml), hydrocortisone (0.5 μg/ml), and epidermal growth factor (20 ng/ml). MDA-MB-231 and MCF7 were grown in DMEM supplemented with 10% FBS.

Antibodies. Monoclonal antibodies to proMMP-9 (CA-209) were described previously (41 and Appendix). Chain-specific monoclonal antibodies to human type IV collagen were a gift from Dr. Sado and Ninomya (Okayama, Japan).

Iodination of proMMP-9 and TIMP-1. Purified proMMP-9 and TIMP-1 were iodinated using IodoGen (Pierce Chemical Co., Rockford, IL). Briefly, glass vials were coated with 4 µg of Iodogen dissolved in 100% chloroform and dried with a gentle stream of nitrogen gas. Fifty to one hundred micrograms of either proMMP-9 or TIMP-1 in 100 µl collagenase buffer were placed in an Iodogen coated vial and allowed to incubate for 1 min at 25°C. Na^{125}I (500 µCi) was added to each vial and the iodination reaction was allowed to continue for 3 min at 25°C. The reaction was stopped by the addition of 100 µl of a solution containing 200 µg of bovine serum albumin (BSA) and 2 mM NaI in distilled water. Unincorporated Na^{125}I was removed on a 1 ml Sephadex G-50 fine column equilibrated with 50 mM Tris-HCl, pH 7.5 containing 5 mM $CaCl_2$, 150 mM NaCl and 0.02% Brij-35 (collagenase buffer). The specific activity of ^{125}I-proMMP-9 and ^{125}I-TIMP-1 was determined after TCA precipitation and quantitation of protein in Coomassie blue-stained SDS-polyacrylamide gels relative to a standard curve of unlabeled purified proteins. Typically, the specific activity of ^{125}I-TIMP-1 was 3.4 x 10^6 cpm/µg and for ^{125}I-proMMP-9 was 7 x 10^6 cpm/µg. No detectable autocatalytic/degradation forms of MMP-9 were observed in the iodinated enzyme as determined by both gelatin-zymography and autoradiography.

Binding of ^{125}I-proMMP-9 to MCF10A Cells. Monolayers of MCF10A cells grown in 12 well (22 mm) plates were rinsed with cold DMEM supplemented with 0.5% BSA and 25 mM HEPES pH 7.5 (binding media) and incubated in binding media for 15 min at 4°C. The binding media was aspirated and various concentrations of ^{125}I-proMMP-9 diluted in binding media were added to each well (300 µl/well) in triplicates in the presence or absence of 80-fold excess unlabeled proMMP-9. After 30 min incubation (4°C), the media was aspirated and the cells were washed 3 times with cold PBS containing 0.1% BSA. The cells were then lysed with 0.5 ml/well of 1 M NaOH for determination of radioactive counts in a Packard gamma counter (Model 5650) and results were expressed as a mean of values obtained for triplicate samples. Cell number was determined in parallel wells. Time course experiments were similarly performed, except that the concentration of ^{125}I-proMMP-9 for each well was kept constant at 18 nM and the cells were harvested after various time intervals at 4°C. The specific binding of ^{125}I-proMMP-9 was calculated as: proMMP-9 specific binding (fmol) = fmol total ^{125}I-proMMP-9 bound minus fmol ^{125}I-proMMP-9 plus unlabeled proMMP-9 bound. Typically, specific binding represented an average of approximately 20-30% of total bound ^{125}I-proMMP-9. The association rate constant (k_{on}) of proMMP-9 was determined from the time course experiment following logarithmic transformation of the amount of specifically bound ^{125}I-proMMP-9 *versus* time. Where $A_t = A_O e^{-Kt}$, it follows that $\ln (A_O / A_t) = Kt$ and therefore, the k_{on} is determined from the slope of a line plotted as ln([LReq]/[LReq]-[LR]) *versus* time. The slope of this line was determined by linear regression analysis using Microsoft Excel™ and the error represents the standard deviation of the slope. The equilibrium binding constant (K_d) and the number of binding sites per cell were determined following Scatchard transformation of the data. The slope and the intercept from the Scatchard analysis were determined by linear regression analysis using Microsoft Excel™ and the error represents the standard deviation of the slope and intercept. All binding studies were repeated at least 3 times.

Coupling of MMP-9 to Affi-Gel 10. Purified MMP-9 (1 mg) in 50 mM HEPES pH 7.5 was allowed to bind to 1 ml Affi-Gel 10 (BioRad) for 5 h at 4°C in the presence of 60 mM $CaCl_2$. After the coupling was complete, the beads were briefly centrifuged (1000 x g) and the supernatant was subjected to SDS-PAGE to determine the amount of uncoupled MMP-9 using known amounts of purified MMP-9 as standard. This analysis demonstrated that >99% of the available MMP-9 was coupled to the Affi-Gel 10 matrix, designated Affi-Gel 10-proMMP-9. The immobilized proMMP-9 maintained its capability to bind TIMP-1 as determined by binding of ^{125}I-TIMP-1 compared to soluble enzyme (data not shown).

Affinity Purification of pro-α2(IV) . All purification procedures were performed at 4°C. A lysate of MCF10A cells in lysis buffer (~40 ml, 132.5 mg of protein) was incubated with 1 ml of Affi-Gel 10-MMP-9 matrix batchwise overnight. The Affi-Gel 10-proMMP-9 matrix was collected by pouring the lysate-Affi-Gel 10-proMMP-9 mixture into a 10 ml polyprep column (BioRad) and the flow through fraction collected. The matrix was then washed with 20 ml of 25 mM Tris pH 7.5, 500 mM NaCl, 0.1% NP-40, 2 mM PMSF, 5 mM benzamidine, 10 µg/ml leupeptin and 10 µg/ml aprotinin. This fraction was designated wash 1. The column was then washed with 10 ml of the same buffer as described above but containing 150 mM NaCl. This fraction was designated wash 2. The 190-kDa protein (pro-α2(IV)) was eluted from the column with 4.5 ml of 50 mM Tris pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 20% DMSO, 2 mM PMSF, 5 mM benzamidine, 10 µ/ml leupeptin and 10 µg/ml aprotinin. Three 1.5 ml fractions of eluate were collected. Forty microliters of the load, flow through, wash 1, wash 2 and eluate fractions were analyzed by ligand blot as described above. In addition, these fractions were analyzed by SDS-PAGE and silver staining. The protein concentrations of each column fraction were determined by the BCA Protein Assay (Pierce) reagent according to the manufacturer's instructions..

Surface Biotinylation of MCF10A cells. Cell surface proteins were biotinylated with 0.5 mg/ml sulfo-NHS-biotin (Pierce) for 30 min at 4°C in PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS-CM). The reaction was quenched with freshly prepared 50 mM NH_4Cl in PBS-CM followed by three washes with cold PBS-CM. The cells were then lysed with 2 ml of ice-cold lysis buffer: 25 mM Tris buffer pH 7.5, 100 mM NaCl, 1% NP-40, 10 µg/ml aprotinin, 1 µg/ml leupeptin, 5 mM benzamidine, and 1 mM phenylmethylsulfonylfluoride (PMSF) per 150 mm culture plate. The lysates were incubated for 1 h on ice followed by a 15 min centrifugation (13,000 x g) at 4°C. The supernatant was collected and analyzed immediately for the presence of MMP-9-binding proteins as described below.

Affi-Gel 10-proMMP-9 Affinity Chromatography. Lysates (0.5-1 ml) of non-biotinylated or biotinylated MCF10A cells were incubated with either 50 µl Affi-Gel 10-proMMP-9 or uncoupled Affi-Gel 10 matrix overnight at 4°C with rotation. After incubation, the beads were washed with cold 50 mM Tris buffer pH 7.5, 150 mM NaCl, 0.1% NP-40 and 10% glycerol followed by one wash in the same buffer containing 500 mM NaCl and three washes in the same buffer but containing 150 mM NaCl. After a brief centrifugation, the bound proteins were eluted with 40 µl of cold collagenase buffer containing 10 % DMSO and subjected to SDS-PAGE under reducing conditions followed by blotting to a BA-S 85 nitrocellulose membrane (Schleicher & Schuell, Keene, NH). After blocking (12 h, 4°C) with 3% BSA and 3% nonfat dry milk in 100 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.02% NaN_3 (blotto), the blots were washed twice with T-TBS (20 mM Tris buffer, pH 7.5, containing 137 mM NaCl and 0.1% Tween-20). Detection of the proMMP-9 binding proteins was accomplished by streptavidin-HRP or by ligand blot analysis using the enhanced chemiluminescence (ECL) kit (Amersham, Arlington Heights, IL) according to the manufacturer's instructions.

Ligand Blot Analysis. Detection of the proMMP-9-binding proteins after the affinity step was also accomplished by ligand blots analysis. The nitrocellulose membranes containing the eluted fractions were incubated (1 h, 25°C) with 1 μg/ml of purified proMMP-9 in T-TBS containing 0.5% non-fat dry milk and 1% NP-40. The blots were then washed several times with T-TBS followed by an incubation (1 h, 25°C) with T-TBS containing 0.2 μg/ml of a monoclonal antibody to MMP-9 (CA-209) and 0.5% non-fat dry milk. After three washes in T-TBS the blots were incubated with the secondary anti-mouse-HRP antibody and developed with the ECL kit.

Co-immunoprecipitation of proMMP-9-binding Protein with proMMP-9. Lysates of surface-biotinylated or non-biotinylated MCF10A cells (2 ml/150 mm plate) in lysis buffer were incubated with 0.5-1μg/ml of purified proMMP-9 for 1 h at 4°C. Then, 5 μg of the appropriate antibody or control IgG were added for another 16 h incubation at 4°C. Each sample received 50 μl of Protein G Sepharose 4 Fast Flow beads (Pharmacia) followed by an additional incubation for 3 h at 4°C with continuous rocking. The beads were then washed with cold 50 mM Tris buffer pH 7.5, 150 mM NaCl, 0.1% NP-40 and 10% glycerol followed by one wash in the same buffer supplemented with 500 mM NaCl and three additional washes with the same buffer but containing 150 mM NaCl. The beads were boiled in the presence of 20 μl of Laemmli sample buffer under reducing conditions and, after a brief centrifugation, the supernatants were subjected to SDS-PAGE and blotting. Detection was accomplished with streptavidin-HRP and by ligand blot analysis.

Analysis of the Affinity of pro-α2(IV) for proMMP-9 and proMMP-2. Affinity purified pro-α2(IV) (180 ng) was allowed to complex with purified and ^{35}S-proMMP-2 (0. 667 μM and 114,146 cpm/pmol) or ^{35}S-proMMP-9 (0.445 μM and 21,196 cpm/pmol) for 1 h at 4^{O} C. The samples were then subjected to Superose 6 gel filtration column chromatography equilibrated with 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, and 0.02% Brij-35 at a flow rate of 0.2 ml/min and 70 fractions (0.3 ml) were collected. Aliquots (0.2 ml) of each fraction were placed in 5 ml of scintillation fluid and counted for 4 min. Complex formation was evaluated based upon the relative inclusion of ^{35}S-proMMP-2 and ^{35}S-proMMP-9 alone or in complex with pro-α2(IV) and the pmols of proMMP-2 and proMMP-9 complexed with pro-α2(IV) were calculated.

6.2 RESULTS

6.2.1. Analyses of proMMP-2 Activation in Primary Breast Fibroblasts.

Please, note that the Figures are included separately after the References.

Effect of Collagen I on proMMP-2 Activation in Primary Breast Fibroblasts. We first analyze the ability of the various isolated fibroblasts to activate proMMP-2 in response to TPA and ConA. As shown in **Figure 1**, ConA (lane 2) induced proMMP-2 activation in all the fibroblasts except in WS9T. The effect of TPA on proMMP-2, however, was less predictable and some fibroblasts did not respond to TPA. We also found no direct correlation between the level of proMMP-2 activation and whether the fibroblasts were isolated from an area closely associated with tumor (T) or from a distant area (B). All attempts to induce proMMP-2 activation by co-culture with breast cancer cell lines or by conditioned media from breast cancer cells, resulted in inconsistent results with little or

no effects. In other studies, we have found no effects of growth factors (bFGF, EGF, TGF-β, IL-1) on proMMP-2 activation.Therefore, we abandoned these inconsistent experiments and we decided to examine the effects of collagen I substrate. **Figure 2**, shows a gelatin zymogram of a typical experiment with WS12B and WS12T cultured on various amounts of collagen I (Fig. 2, lanes 4-9). As little as 0.2 mg of collagen I induced maximal proMMP-2 activation (Fig. 2, lane 4). In contrast to collagen I, fibronectin was unable to induce proMMP-2 activation (Fig. 2, lane 3) even at high concentrations (100 μg/cm^2) (data not shown). These studies show that interactions of breast fibroblasts with collagen I induces the activation of proMMP-2.

Pulse Chase Analysis of MT1-MMP and MMP-2 in Breast Fibroblasts Cultured in Collagen I. We next examined the effect of collagen I and ConA on the biosynthesis of MT1-MMP and MMP-2 by pulse chase analysis of media and cell lysates.As shown in **Figure 3A**, MT1-MMP is rapidly synthesized in WS12B cells as ~63-kDa precursor form that is rapidly processed to a 57-kDa form, shown by the arrow. No significant differences were found between untreated and ConA-treated cells in terms of processing of MT1-MMP. However, with ConA we detected a somewhat increase in MT1-MMP protein in WS12B fibroblasts (**Fig. 3A**). A similar pattern of biosynthesis and processing was observed in WS12T cells (**Fig. 3C**). Thus, although ConA induces proMMP-2 activation, this process appears to be independent of the processing of MT1-MMP. In cells cultured on collagen I (**Fig. 3B**), we observed similar processing of MT1-MMP than cells seeded on plastic. However, on collagen I, the MT1-MMP protein level were higher suggesting that the effect of collagen I on proMMP-2 may be the result of MT1-MMP synthesis or stability. It should be mentioned that we do not know whether the 57-kDa represent the active form of MT1-MMP or whether the enzyme is on the cell surface. These issues are now under investigation.

The biosynthesis and secretion of MMP-2 was examined in the cell lysates and in the media. As shown in **Figure 4**, MMP-2 is already detected in the media after a 30 min chase period regardless of the type of treatment (ConA, collagen I, plastic). The enzyme was detected as major band of 72-kDa possibly representing the latent form even though the zymogram (**Fig. 1**) showed presence of active MMP-2. The reasons for this discrepancy are (i) that the enzyme is not completely activated by these treatments, (ii) the high specific activity of the active form in zymograms and (iii) the low amounts of radioactive MMP-2 precipitated by the antibodies even though the antibodies used bind to both latent and active enzyme. The expression of MMP-2 in the media demonstrated lower levels of enzyme after ConA treatment or growth in collagen I possibly due to the binding of the enzyme to the cell surface for activation. Indeed, when the enzyme was examined in the cell lysate we observed a significant difference between collagen and plastic and between cells treated or not with ConA with higher amounts of enzymes precipitated from the cells grown on collagen I or treated with ConA. From these studies we suggest that induction of proMMP-2 activation with either ConA or with a collagen I gel result in an increased cell association of MMP-2. We are now investigating the presence of MMP-2 on the surface in response to these treatments. Also, the biosynthesis of TIMP-2 will be examined.

Norther Blot Analyses of the mRNA Expression of MT1-MMP, MMP-2 and TIMP-2. To determine if the effect on proMMP-2 activation by collagen I or ConA treatment was transcriptionally regulated , we examined the levels of mRNA levels using specific cDNA probes. These results are shown in **Figure 5** for both WS12B and WS12T fibroblasts. The blots were analyzed by densitometry using the signal of GAPDH for normalization. **Table 1** shows the quantitative analyses obtained. It was found that neither MMP-2 nor TIMP-2 mrNA levels were significantly altered by these treatments. In contrast, both collagen I and ConA induced the

expression of MT1-MMP mRNA by approximately 2-folds consistent with the higher levels of MT1-MMP protein observed in the pulse chase analysis (**Fig. 3**).

Table 1
Effect of Collagen I and ConA on mRNA Levels of MT1-MMP, MMP-2 and TIMP-2 in Primary Breast Fibroblasts.

Cell	WS12B			WS12T		
	Plastic	conA	Collagen I	Plastic	conA	Collagen I
MT1-MMP	1.0	2.0	2.5	1.0	1.6	2.2
MMP-2	1.0	1,19	1.09	1.0	1.02	1.03
TIMP2	1.0	1.0	1.0	1.0	1.12	1.16

The above studies demonstrate the importance of cell-matrix interactions in the regulation of proMMP-2 activation in breast fibroblasts. We suggest that during the progression of breast cancer into more malignant phenotypes, the stroma cells in response to a desmoplastic process that is characteristic of breast tumors, the fibroblasts in the tumor stroma are induced to produce MT1-MMP which in turn induces the activation of proMMP-2. These two enzymes can cleave many ECM components and therefore may contribute to the invasive abilities of breast cancer cells. It is still unknown how the stroma cells, which are the producers of these enzymes, as determined by *in situ* hybridization studies, are then utilized by the tumor cells since the proteins are found on the breast cancer cells. This remains the major mystery in the understanding of the role of MMPs in breast cancer progression. However, significant advances have been made on the mechanisms regulating the expression and activation of these enzymes. Further studies are required to examine the role of TIMP-2 in these processes. In agreement with the objectives of this grant, we will continue to examine these processes using the primary breast fibroblasts and various breast cancer cell lines. The above studies are now being prepared for publication.

6.2.2 Surface Binding of ProMMP-9 to Breast Epithelial Cells.

Another important aspect of breast cancer progression and proteases, is the reported association of MMP-9 with the surface of breast cancer cells while the mRNA has been mostly localized in stromal cells (fibroblasts, macrophages, endothelial cells). Thus, the mechanism of cell surface association of MMP-9 in breast epithelial remains unknown In our previous report, we described the association of proMMP-9 with MCF10A cells, an immortalized cell line derived from a woman with fibrocystic disease of the breast. The results reported were accepted for publication in Cancer Research and will be published in the August 1 issue this year (please, find enclosed a copy of the proofs). Based on these findings, we decided to unveil the mechanism of association in order to determine whether there is a surface component responsible for the binding of MMP-9. In this report, we inform about these latest studies which resulted in the identification of the pro-α2(IV) chain of collagen IV as the major surface protein MCF10A cells involved in the binding of proMMP-9.

Characteristics of ^{125}I-proMMP-9 Binding to MCF10A Cells. Previously we showed that while MCF10A cells produce very low amounts of proMMP-9, exposure of the cells to TPA results in

the secretion of proMMP-9 that associates to the cell surface (Appendix). To characterized the binding parameters of proMMP-9 to MCF10A cells, we carried out binding assays of radioiodinated enzyme to untreated MCF10A cells to avoid interference with the endogenously produced proMMP-9. **Figure 6A** shows time dependent, saturable and specific binding of ^{125}I-proMMP-9 to MCF10A cells at 4°C. Logarithmic transformation of the data revealed a k_{on} value of $6.94 \pm 0.67 \times 10^4$ M^{-1} s^{-1} (Figure 6, inset). Incubation of MCF10A cells with increasing concentrations (1-40 nM) of ^{125}I-proMMP-9 in the presence of 80-fold excess of unlabeled enzyme demonstrated a specific saturable binding (**Figure 6B**). Scatchard analysis revealed a K_d value of $2.16 \pm 0.16 \times 10^{-8}$ M. The linearity of the Scatchard analysis was reflective of an homogeneous population of approximately 130,000 high affinity binding sites per cell (**Figure 6B**, inset). Analysis of bound ^{125}I-proMMP-9 after a 2 h incubation revealed presence of the monomer and dimer forms and no indication of active species as determined by autoradiography after SDS-PAGE analysis or by gelatin zymography (data not shown). It should be mentioned that binding of active MMP-9 to MCF10A cells was impractical due to the autocatalytic conversion of the purified 82-kDa active species to the 67-kDa forms during the iodination procedure.

Identification of a 190-kDa Cell Surface proMMP-9-Binding Protein. The above binding data was consistent with the existence of a single proMMP-9-binding component on the surface of MCF10A cells. To identify the putative proMMP-9-binding protein, we carried out an affinity purification procedure using an Affi-Gel 10 matrix that was covalently linked with human recombinant proMMP-9 (Affi-Gel 10-proMMP-9). Lysates of surface-biotinylated MCF10A cells were then incubated with Affi-Gel 10-proMMP-9 affinity matrix or uncoupled Affi-Gel 10 matrix and the bound proteins were eluted with 10% DMSO in collagenase buffer. The proMMP-9-bound proteins were then subjected to SDS-PAGE and blotting. Detection was accomplished by either streptavidin-HRP or ligand blot analysis as described under "Experimental Methods." **Figure 7** shows that MCF10A cells express a major 190-kDa protein that specifically binds to the Affi-Gel 10-proMMP-9 matrix (Fig. 3, lanes 2, 4 and 5). This protein was not detected in cell lysates incubated with uncoupled Affi-Gel 10 matrix (Fig. 3, lane 1 and 3). The 190-kDa protein was recognized by streptavidin-HRP (Fig. 3, lane 2, avidin-HRP panel) consistent with cell surface localization. The avidin recognition was specific since blots of samples derived from cell lysates of non-biotinylated MCF10A cells and incubated with Affi-Gel 10-proMMP-9 were negative (**Fig. 7**, lane 4, avidin-HRP). The presence of the 190-kDa protein in the non-biotinylated MCF10A cells was confirmed by ligand blot analysis (**Fig. 7**, lane 4, ligand blot). The electrophoretic migration of the 190-kDa protein was similar under nonreducing (**Fig. 7**, lane 5) or reducing conditions (**Fig. 7**, lanes 2 and 4). In contrast, under nonreducing conditions, MMP-9 exhibited the classical presence of monomer (92 kDa) and homodimer forms (>210 kDa) (**Fig. 7**, lane 6), as expected. Collectively, these results demonstrate that MCF10A cells express a 190-kDa cell surface protein that is capable of binding proMMP-9.

Co-immunoprecipitation of a proMMP-9/190-kDa Complex. To determine whether the 190-kDa cell surface protein could form a complex with proMMP-9, a co-immunoprecipitation experiment was carried out following addition of proMMP-9 to a lysate of surface-biotinylated MCF10A cells. The mixture was then immunoprecipitated with anti-MMP-9 antibodies and protein-G-Sepharose beads. The immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose membranes and developed with streptavidin-HRP or by ligand blot analysis. **Figure 8** (avidin-HRP) shows that although many surface-biotinylated proteins were non-specifically absorbed to the protein-G-Sepharose beads, a 190-kDa cell surface biotinylated protein was consistently and specifically immunoprecipitated by anti-MMP-9 antibodies in the presence of exogenous proMMP-

9. The 190-kDa protein could not be detected in the absence of enzyme or when non-immune rabbit IgG was used instead of anti-MMP-9 antibodies (**Fig. 8**, avidin-HRP). Additional data supporting the specificity of the precipitation of the proMMP-9/190-kDa complex was obtained when the same blot was subjected to ligand blot analysis (**Fig. 8**, ligand blot). These results showed that only the surface-biotinylated 190-kDa protein and proMMP-9 could be detected in the samples precipitated with enzyme and anti-MMP-9 antibodies.

Affinity Purification of the 190-kDa MMP-9 Binding Protein. The 190-kDa protein was purified from lysates of MCF10A cells using the Affi-Gel 10-proMMP-9 matrix as described in the "Experimental Methods". This procedure resulted in the isolation of major 190-kDa protein, as determined by SDS-PAGE and silver staining (**Fig. 9A**) and ligand blot analysis (**Fig. 9B**) of the eluted fractions. The purified 190-kDa protein showed a similar molecular mass under both reducing and nonreducing conditions. Analysis of the yield obtained after purification indicated an approximately 3300-fold purification of the 190-kDa protein by this procedure.

The 190-kDa proMMP-9-Binding Protein.is the pro-α2(IV) Chain of Collagen IV. Pooled fractions from the proMMP-9 affinity column containing the 190-kDa protein were subjected to preparative SDS-PAGE and send for microsequencing. Analyses of three HPLC-purified peptides obtained after tryptic digestion revealed the following amino acid sequences. Peptide 1, GVSGFPGADGIPGHPGQGGP; peptide 2, DGYQGPDGPRG and peptide 3, KIAIQPGTVGPQG. Analysis of these sequences by the BLAST program revealed that they correspond to residues 109-128, 325-334 and 1396-1408, respectively, of the human procollagen α2(IV) chain of collagen IV. To further demonstrate that the purified 190-kDa protein was indeed pro-α2(IV), three monoclonal antibodies (H22, H25 and H21) to human pro-α2(IV) were found to react with the protein (**Figure 10A**). Furthermore, co-immunoprecipitation of the 190-kDa protein from a MCF10A lysate with exogenous proMMP-9 using an anti-MMP-9 antibody followed by immunoblot analysis with H22 demonstrated that the coprecipitated 190-kDa protein was indeed the pro-α2(IV) chain (**Figure 10B**).Taken together, these studies demonstrated that the major proMMP-9 binding protein in MCF10A cells is the pro-α2(IV) chain of collagen IV.

Affinity of proMMP-9 and proMMP-2 for pro-α2(IV). Since proMMP-9 bears a high degree of sequence similarity with proMMP-2 and both enzymes contain a gelatin-binding domain, we wished to determine whether proMMP-2 also binds to the pro-α2(IV) chain. To this end, we carried out a competition and co-immunoprecipitation experiment using purified pro–α2(IV). It should be mentioned that pro-α2(IV) could not be detected by ligand blot analysis after incubation with active MMP-2 (data not shown) suggesting that pro-α2(IV) is also a substrate for MMP-2. Therefore, the following experiments were conducted in the presence of 10 mM EDTA to prevent degradation of pro-α2(IV) that may occur by the presence of low amounts active MMP-2 in the proMMP-2 preparation. As shown in **Figure 11**, pro-α2(IV) readily forms a complex with MMP-9 that coprecipitates with anti-MMP-9 antibodies, as determined by ligand blot analysis (**Fig. 11A**, lane 2. In the absence of enzyme, the pro-α2(IV) chain was not detected, as expected (**Fig. 11A**, lane 1). We next tested the ability of proMMP-2 to compete with proMMP-9 for the binding of pro-α2(IV) using either equal molar amounts (**Fig. 11A**, lane 3) or 5-fold molar excess proMMP-2 (**Fig. 11A**, lane 4) in the reaction mixtures. The samples were then subjected to immunoprecipitation with anti-MMP-9 antibodies and resolved by ligand blot analysis. These experiments showed no significant differences in the intensity of the pro-α2(IV) band when the immunoprecipitation was performed in the absence (**Fig. 11B**, lane 2) or in the presence of 5-fold excess molar concentration of proMMP-2 (**Fig. 11B**, lane 4). Furthermore, incubation of

proMMP-2 with pro-α2(IV) in the absence of proMMP-9 followed by immunoprecipitation with two distinct monoclonal antibodies to MMP-2 (CA-801 and CA-805) failed to coprecipitate the pro-α2(IV) chain as determined by ligand blot analysis (**Fig. 11A**, lane 6). As expected, proMMP-2 in the immunoprecipitates could not be detected by the ligand blot technique using proMMP-9 as a probe. However, when the same blot was developed with anti-MMP-2 antibodies, proMMP-2 was readily detected (**Fig. 11B**, lane 8). Same results were obtained when lysates of MCF10A cells were used as a source of pro-α2(IV) chain (data not shown). Taken together these results suggest that the pro-α2(IV) chain exhibits a preferential binding to proMMP-9 in the presence of excess molar concentrations of proMMP-2.

To obtain a quantitave measurement of the relative affinities of proMMP-9 and proMMP-2 to the pro-α2(IV) chain, we examined the ability of purified pro-α2(IV) to form a complex with either ^{35}S-proMMP-2 or ^{35}S-proMMP-9. To this end, pro-α2(IV) (~30 nM) was incubated with increasing concentrations of proMMP-9 (2-250 mM) or proMMP-2 (10-750 nM). The complexes were then subjected to gel filtration and the equilibrium constants (K_d) were determined as described under "Experimental Methods. **Figure 12** shows that both proMMP-9 and proMMP-2 bind to pro-α2(IV) as a function of enzyme concentration. Under these conditions, maximal binding was observed at concentrations of enzymes of 250 nM for proMMP-9 and 750 nM for proMMP-2. From these data, the K_d values of pro-α2(IV) for proMMP-9 and proMMP-2 were calculated to be 45 nM and 350 nM, respectively. This demonstrates that proMMP-9 binds to pro-α2(IV) with an affinity ~8-fold greater than that of proMMP-2 consistent with the results obtained in the co-precipitation experiments.

Specificity of the Binding of ^{125}I-proMMP-9 to Surface-Bound pro-α2(IV). To determine whether the cell surface binding of ^{125}I-proMMP-9 to MCF10A cells was mediated by pro–α2(IV), we used two different experiments. First, ^{125}I-proMMP-9 was preincubated with affinity purified pro–α2(IV) before the cell-binding assays (**Table 2**) and second, the cells were incubated (30 min) with active MMP-9 before the binding assay (**Table 3**). The rationale for the latter experiment was based on the ability of active MMP-9 to hydrolyze pro–α2(IV). As shown in **Table 2**, preincubation of ^{125}I-proMMP-9 with pro–α2(IV) inhibited specific binding by 61% in comparison of enzyme alone.

Table 2

Effects of purified pro-α2(IV) on the binding of ^{125}I-proMMP to MCF10A cells.

^{125}I-proMMP-9 was incubated (1.5 h at 4°C) with a 2-fold molar excess of purified pro-α2(IV) to form a complex. Binding to MCF10A cells was carried out using ^{125}I-proMMP-9 alone or ^{125}I-proMMP-9/pro-α2(IV) complex as described under "Experimental Methods".

Competitor	Specific ^{125}I-MMP-9 Bound
	(fmol / 1.3 x 10^5 cells)
None	12.3
pro-α2(IV)	4.8

Thus, preincubation of ^{125}I-proMMP-9 with the affinity purified pro-α2(IV) inhibited binding to MCF10A cells. As shown in **Table 3** (below) in pretreatment of MCF10A cells with active MMP-9, abrogated (~99%) the specific binding of ^{125}I-proMMP-9. In the presence of TIMP-1, the effect of the active MMP-9 on ^{125}I-proMMP-9 binding was significantly reduced suggesting that the inhibitor interfered with the catalytic activity of MMP-9 (**Table 3**). Ligand blot analysis of MMP-9-treated and untreated MCF10A cells showed a significant reduction on pro–α2(IV) as determined by densitometric analysis of the blots (data not shown) suggesting that the surface-bound pro-α2(IV) is accessible to the catalytic activity of MMP-9.

Table 3

Effect of active MMP-9 on the binding of ^{125}I-proMMP to MCF10A cells.

MCF10A cells in 35 mm wells were washed with binding media and then incubated (45 min, 37°C) with either 1 pmol/well of purified 82-kDa active form of MMP-9 or with active MMP-9 that was previously incubated (30 min, 25°C) with TIMP-1 (2 pmol). As control, some wells of MCF10A cells were incubated with binding media alone. The cells were then washed with binding media and the specific binding of ^{125}I-MMP-9 was determined as described under "Experimental Methods". Similar results were obtained in two independent experiments.

Treatment With	Specific ^{125}I-MMP-9 Bound
	(fmol / 1.3 x 10^5 cells)
No enzyme	16.4
Active MMP-9	1.1
Active MMP-9 + TIMP-1	9.84

Thus, the experiments in Table 2 and 3 demonstrate that the binding of proMMP-9 to MCF10A cells is mediated by surface-associated pro–α2(IV).

ProMMP-9 Binding in Malignant Breast Cancer Cells. We wished to determine whether breast cancer cell lines can bind proMMP-9. To this end, we carried out binding assays with ^{125}I-proMMP-9 as described in the "Experimental Methods" using the metastatic breast cancer cell line MDA-MB-231. These results are shown in **Table 4** and compared with the binding parameters of MCF10A cells.

Table 4

Comparison of the Binding Parameters of ^{125}I-proMMP-9 to MCF10A and MDA-MB-231 Cells

Cell Line	K_d	K_{on}	Sites/Cell
MCF10A	2.16 ± 0.16 x 10^{-8} M	6.94 ± 0.67 x 10^4 M^{-1} s^{-1}	1.3 ± 0.08 x 10^5
MDA-MB-231	2.18 ± 0.23 x 10^{-8} M	6.65 ± 0.53 x 10^4 M^{-1} s^{-1}	0.12 ± 0.02 x 10^5

As shown in **Table 4**, we have found that MDA-MB-231 cells bind ^{125}I-proMMP-9 with an affinity similar to that observed with MCF10A cells. However, these cells showed a reduced number of sites. The reason for the reduced number of sites in MDA-MB-231 cells is under investigation but may involved degradation of cell surface pro-α2(IV chain in the malignant cells.

Expression of ProMMP-9 Binding Proteins in MDA-MB-231 and MCF7 Breast Cancer Cells. The existence of proMMP-9 binding proteins in these cells was determined using a co-immunoprecipation experiment with exogenous proMMP-9 an anti-MMP-9 antibodies followed by analysis of the immunoprecipitates by ligand blot analysis. As shown in **Figure 13**, both MDA-MB-231 and MCF7 cells express a major proMMP-9 binding protein of ~200 kDa. In addition, the MDA-MB-231 cells showed a specific protein of ~120 kDa. In comparison, MCF10A cells were subjected to the same procedure which demonstrated the presence of pro-α2(IV). Although the precise nature of the 200-kDa protein in the cancer cell lines remains to be determined, it is likely to be the pro-α2(IV) chain showing a slight difference in molecular mass possibly due to differences in glycosilation. The nature of the 120-kDa protein is also unknown. Nevertheless, these studies show that breast cancer cell lines express proMMP-9 binding proteins. The function of these remains to be determined.

These studies demonstrated a unique interaction of proMMP-9 with pro–α2(IV). Interestingly, we have found that proMMP-9 while it binds to monomeric pro–α2(IV), shows little binding to trimeric collagen IV (data not shown) suggesting that the site of interaction between proMMP-9 and pro–α2(IV) is not available in the native collagen IV molecule. The significance of these findings are under investigation. It was interesting to observe that proMMP-2 which is homologous to proMMP-9 showed a significantly weaker affinity for the pro–α2(IV) chain in spite that both enzymes hydrolyze pro–α2(IV). This suggest that the region of proMMP-9 that binds to pro–α2(IV) may be different from that present in proMMP-2. We have evidence that binding is mediated through the gelatin-binding domain since the complex of proMMP-9 and pro–α2(IV) is disrupted by gelatin. Therefore, other unique sites in proMMP-9 and/or the conformation of this enzyme are crucial for the binding to pro–α2(IV). This demonstrate a unique interaction of proMMP-9 with pro–α2(IV). Biologically, the association of proMMP-9 with pro–α2(IV) may help to localize the enzyme on the cell surface or in areas of cell-matrix contacts where degradation of ECM takes place. The importance of this interaction for the invasive abilities of breast cancer cells and the degradation of basement membranes is under investigation.

6.2.3. Expression of Cathepsin B and D in Primary Breast Fibroblast Grown on Collagen I.

Effect of Collagen I on Cathepsin B and D Protein Expression. We examined the effects of collagen I on the expression of cathepsin B and D in WS12B and WS12T breast fibroblasts. As shown in **Figure 14**, collagen I induced a slight increase in the protein level of cathepsin B and D in WS12T cells (lanes 6 and 7). Lanes 4, 5, 8 and 9 show results obtained with WS12B and WS12T cells that were virally transformed to facilitate *in vitro* growth and examined here for expression of cathepsin B and D. These data show a decreased expression of both enzymes in the transformed WS12B cells. We would like to emphasize that the effects of the transformation are unpredictable but was intended to preserve the breast fibroblasts as a cell line since none are available.

Northern Blot Analyses of Cathepsin B in Primary Breast Fibroblasts Grown on Collagen I. As shown in the northern blot analysis of **Figure 15**, collagen I had no effect on the level of cathepsin B mRNA as determined by comparison to the levels of GAPDH mRNA (not shown).

7. CONCLUSIONS (96-97)

1. We have found induction of proMMP-2 activation.in primary breast fibroblasts grown on collagen I gels. Both cathepsin B and D protein levels were slightly increased on collagen I.

2. Pulse chase analysis demonstrated no significant differences in the processing of MT1-MMP or MMP-2. However, higher levels of MMP-2 were associated with the cells.

3. Northern blot analysis demonstrated a 2-fold induction of MT1-MMP mRNA in primary breast fibroblasts cultured on collagen I.

4. ProMMP-9 binds to MCF10A cells with high affinity (K_d=21 nM) and in a specific and saturable manner consistent with the presence of a homogeneous population of receptors. Binding did not result in proenzyme activation or internalization.

5. Ligand blot analysis, affinity purification and co-immunoprecipitation with proMMP-9 resulted on the identification of a 190-kDa surface protein in MCF10A cells that specifically bound to proMMP-9.

6. The 190-kDa protein was purified and microsequenced. This demonstrated that this protein is the pro–α2(IV) chain of collagen IV.

7. ProMMP-9 exhibits a 8-fold higher affinity for pro–α2(IV) than proMMP-2.

8. Competition experiments demonstrated that the binding of proMMP-9 to MCF10A cells is mediated by surface-bound pro–α2(IV).

9. MDA-MB-231 cells bind proMMP-9 with high affinity. Both MDA-MB-231 and MCF7 cells express proMMP-9 binding proteins.

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FIGURE 1

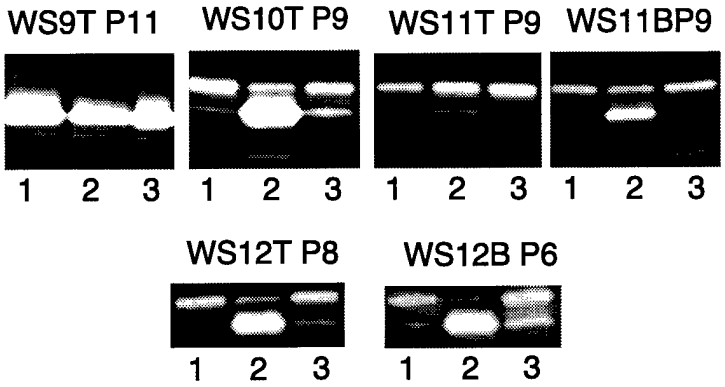

Figure 1. Effect of ConA and TPA on proMMP-2 Activation in Primary Breast Fibroblasts. Cells were incubated with either 100 nM TPA (lane 3), 20 μg/ml of ConA (lane 2) or not treated (lane 1) in serum free media for 12 h. The media was then analyzed by gelatin zymography. Note the conversion of proMMP-2 (72 kDa) to the 65-kDa active formin WS10T, WS11T, WS11B, WS12T and WS12B but not in WS9T.

FIGURE 2

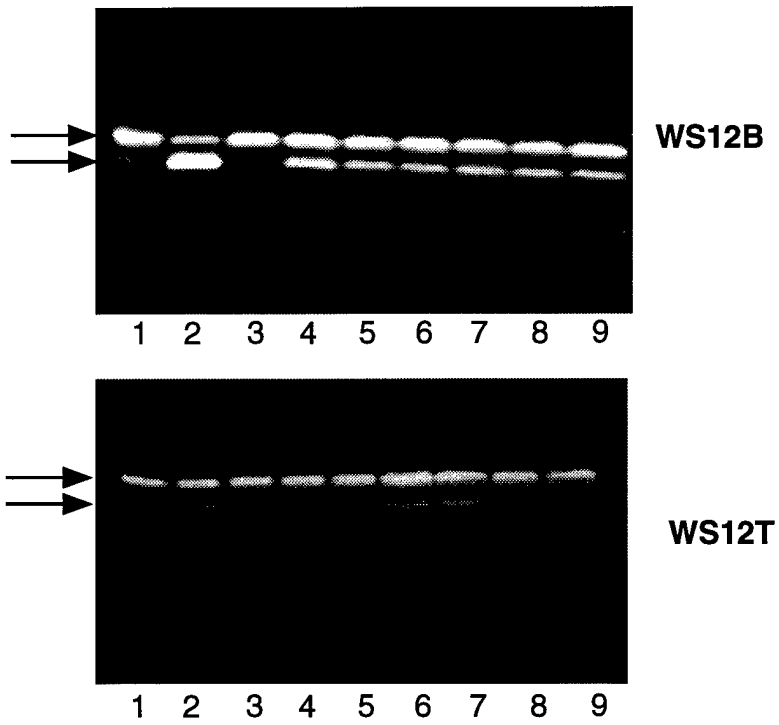

Figure 2. Effect of Collagen I on proMMP-2 Activation. Fibroblast WS12B and WS12T were cultured either on plastic (lane 1), on platic and treated with 20 μg/ml ConA (lane 2), on fibronectin (lane 3, 10 μg/60 mm dish) or in a collagen I gel (lanes 4-9). Lane 4, 0.21 mg; lane 5, 0.316 mg; lane 6, 0.632 mg; lane 7, 1.264 mg; lane 8, 1.89 mg; and lane 9, 2.5 mg/60 mm dish. After 2 days in culture, the media was collected and then analyzed by gelatin zymography. The upper arrow shows proMMP-2 and the lower arrow shows the active MMP-2 (62 kDa).

FIGURE 3

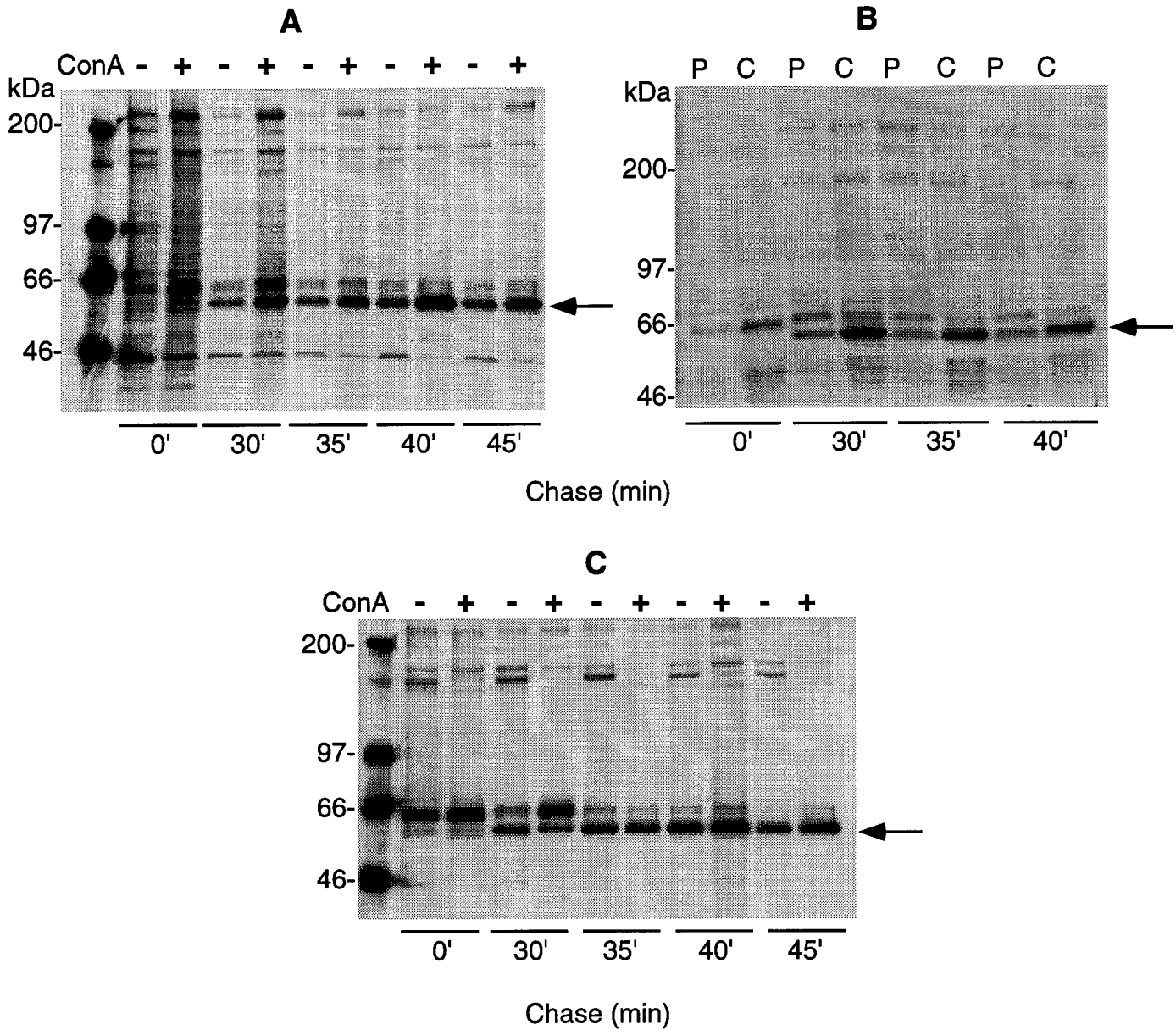

Figure 3. Pulse Chase Analysis of MT1-MMP in Primary Breast Fibroblasts. WS12B (**A** and **B**) and WS12T (**C**) breast fibroblasts were cultured for two days in plastic (P in panel B) or in collagen gels (C in panel B) or treated (+) or not (-) with 20 μg/ml of ConA for 12 hrs. The cells were then pulsed for 15 min with 500 μCi/ml of [^{35}S]methionine. After labeling, the cells were chased for various periods of time as described in the "Experimental Methods" section and the lysates were immunoprecipitated with a polyclonal antibody against MT1-MMP. The arrows demonstrate the 57-kDa MT1-MMP.

FIGURE 4

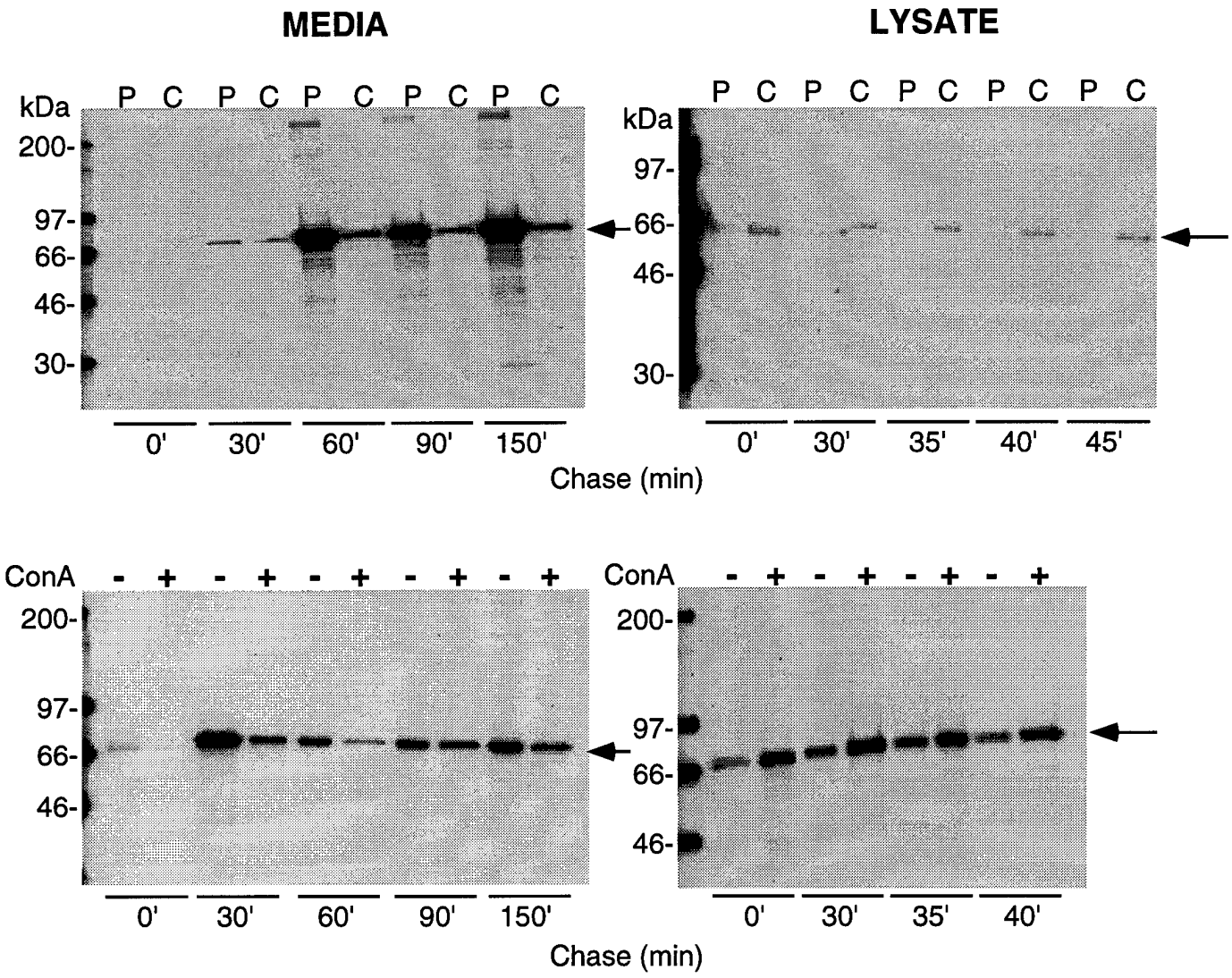

Figure 4. Pulse Chase Analysis of MMP-2 in Primary Breast Fibroblasts. WS12B breast fibroblasts were cultured for two days in plastic (P) or in collagen gels (C) or treated (+) or not (-) with 20 μg/ml of ConA for 12 hrs. The cells were then pulsed for 15 min with 500 μCi/ml of [^{35}S]methionine. After labeling, the cells (LYSATE) and the supernatant (MEDIA) were chased for various periods of time as described in the "Experimental Methods" section and the samples were immunoprecipitated with a monoclonal antibody to MMP-2 (CA-801). The arrows demonstrate the MMP-2. Note the consistent lower amounts of MMP-2 in the media in cells cultured in collagen or treated with ConA and the higher amounts of MMP-2 in the cells grown on collagen or treated with ConA.

FIGURE 5

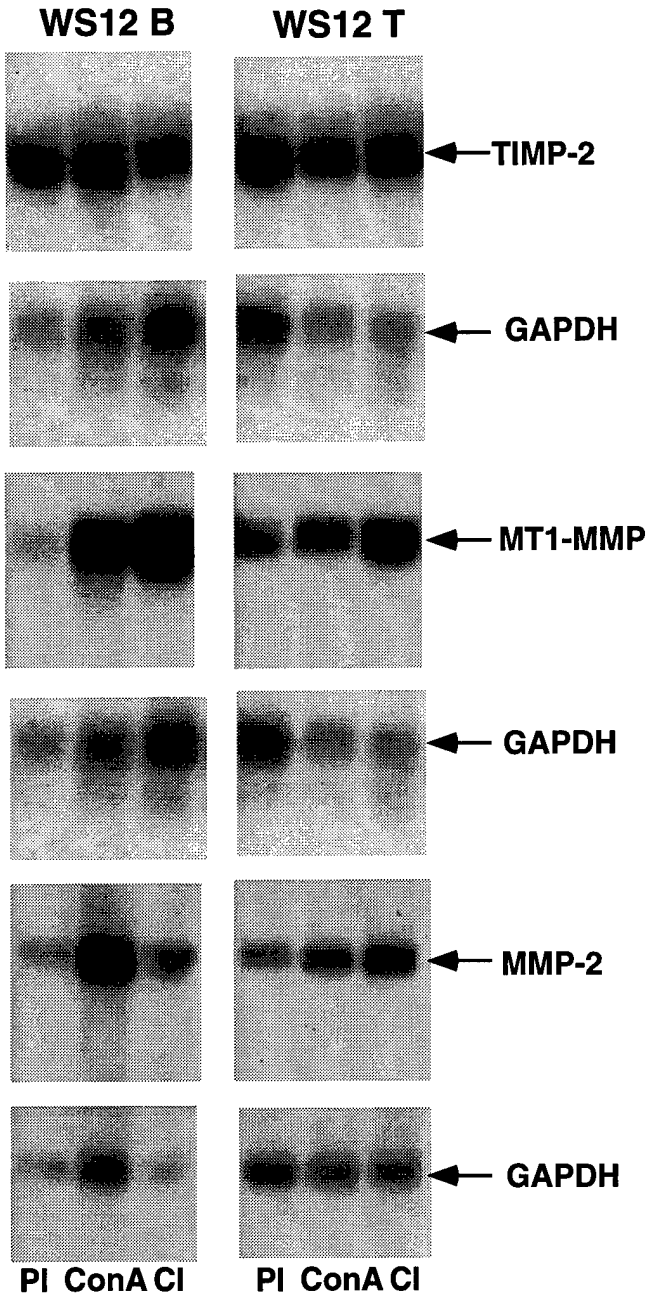

Figure 5. Northern Blot Analyses of TIMP-2, MT1-MMP and MMP-2 in Primary Breast Fibroblasts. WS12B and WS12T (both at passage 7) were cultured on either plastic (Pl), collagen I gel (Cl) or treated with ConA (ConA). Total RNA (5 μg each)was then isolated as described in the "Experimental Methods" section, electrophoresed, blotted and hybridized with the respective cDNA probes. As control, the blots were also hybridized with a GAPDH cDNA probe for normalization of loading.

FIGURE 6

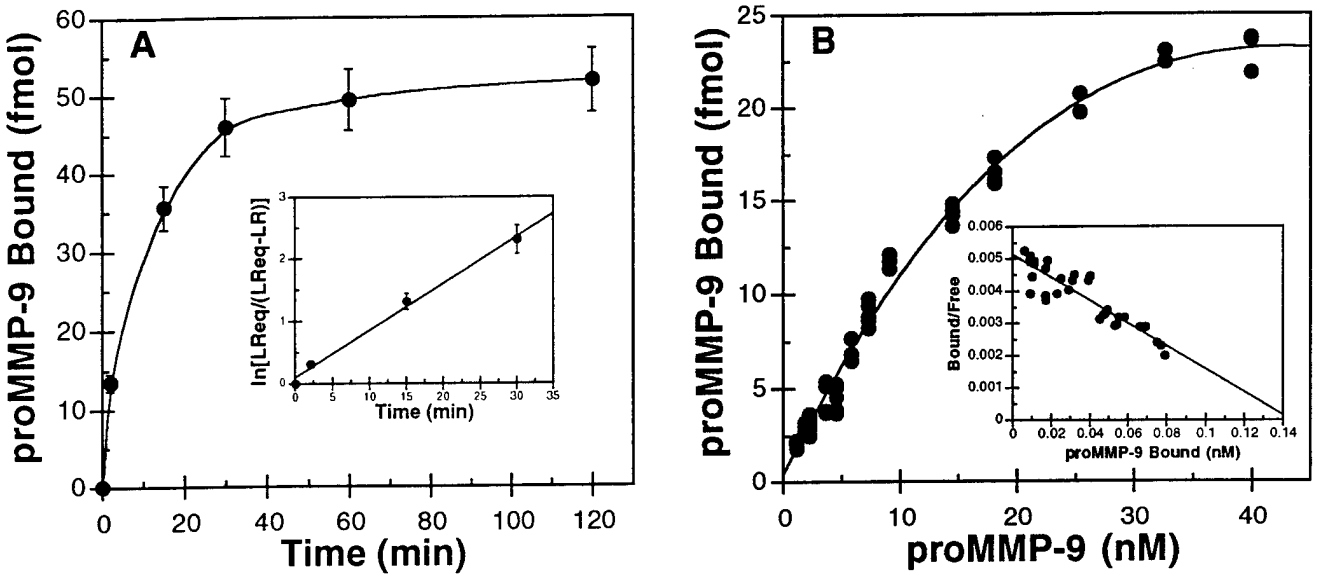

Figure 6. Binding of ^{125}I-proMMP-9 to MCF10A Cells. **A.** Cells (2 x 10^5 cell/well) were incubated with 18 nM of ^{125}I-proMMP-9 for various periods of time (0-120 min) at 4°C. One half of the wells received an 80-fold excess of unlabeled proMMP-9 while the other half received an equivalent volume of binding media. At the indicated time points, the cells were processed as described under "Experimental Methods" to measure the quantity of cell-associated ^{125}I-proMMP-9. Each point represents specific binding (total minus nonspecific) in triplicate wells. A logarithmic transformation of the data is shown (inset) for calculation of the k_{on}. **B.** MCF10A cells (2 x 10^5 cell/well) were incubated (30 min at 4°C) with increasing concentrations (1-40 nM) of ^{125}I-proMMP-9. One half of the wells received an 80-fold excess of unlabeled enzyme while the other half received an equivalent volume of binding media. At the end of the incubation period, the cells were lysed and the radioactivity measured to asses the quantity of cell associated ^{125}I-proMMP-9. Each point represents specific binding (total minus nonspecific) in triplicate wells. Inset shows the Scatchard plot analysis used to calculate the K_d. This experiment was repeated at least three times with similar results.

FIGURE 7

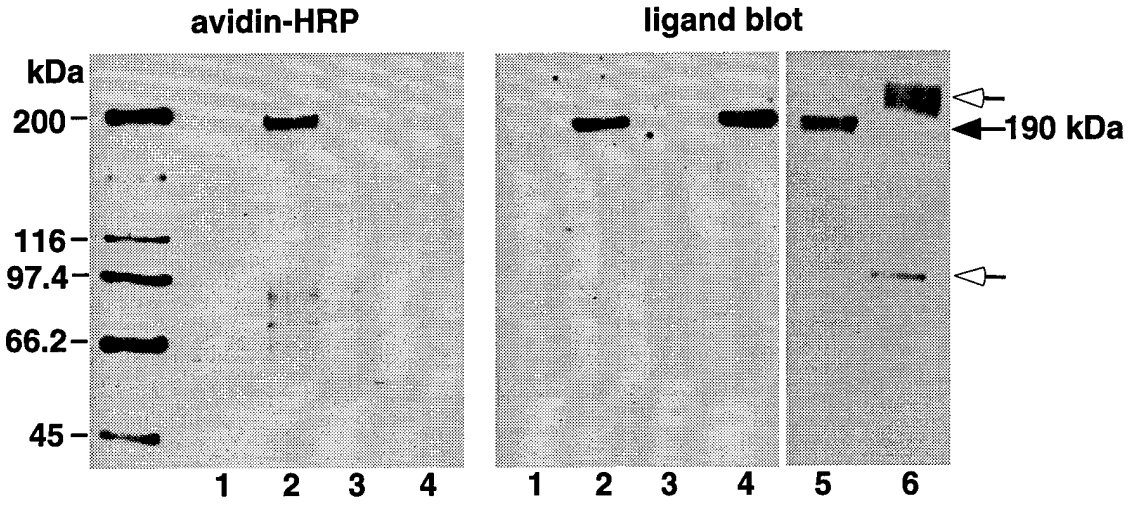

Figure 7. Identification of a 190-kDa MMP-9-Binding Protein with Immobilized proMMP-9. Lysates of surface-biotinylated (lanes 1 and 2) or non-biotinylated (lanes 3 and 4) MCF10A cells were incubated with either Affi-Gel 10-proMMP-9 matrix (lanes 2, 4 and 5) or uncoupled Affi-Gel 10 matrix (lanes 1 and 3) as described under "Experimental Methods". After extensive washes, the bound proteins were eluted with 10% DMSO in collagenase buffer and the eluates were subjected to 4-12% SDS-PAGE either under reducing (lanes 1-4) or non-reducing (lanes 5 and 6) conditions and transferred to a nitrocellulose membrane. Detection of the proMMP-9 binding protein was performed by either streptavidin-HRP (left panel) or ligand blot analysis center and right panels) with purified proMMP-9 (1 μg/ml) as a probe followed by detection with anti-MMP-9 antibody (CA-209) and anti-mouse IgG-HRP using the ECL kit. Lane 6 shows the monomer and dimer forms of recombinant proMMP-9 (10 ng) electrophoresed under non-reducing conditions.

FIGURE 8

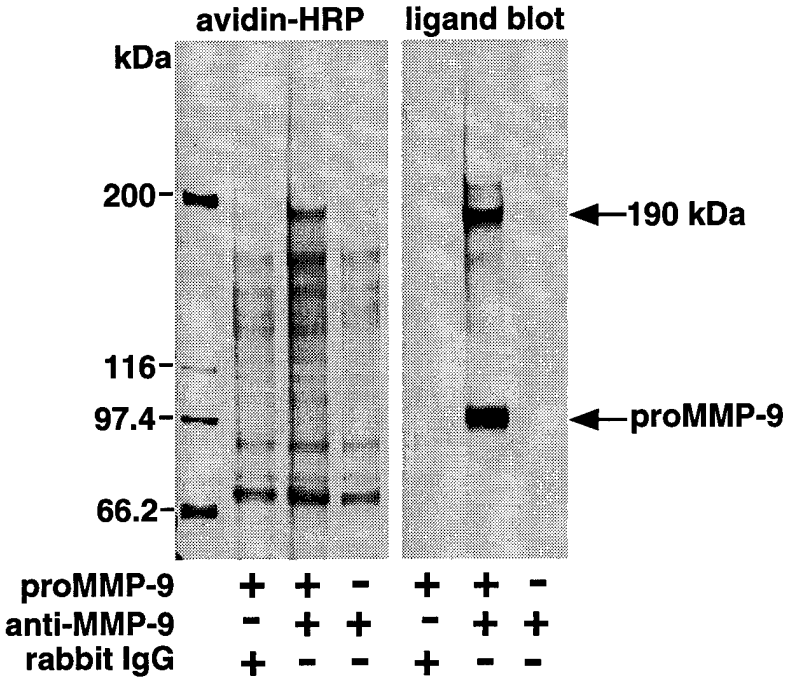

Figure 8. Co-immunoprecipitation of proMMP-9 with the 190-kDa proMMP-9-Binding Protein. Lysates (0.5 ml) of surface-biotinylated MCF10A cells were incubated (+) or not (-) with purified proMMP-9 (1 μg/ml) followed by addition of rabbit polyclonal anti-MMP-9 antibodies (5 μg). Some samples received only rabbit IgG as control. The samples were then incubated with protein-G Sepharose beads as described under "Experimental Methods". The immunoprecipitates were subjected to 4-12% SDS-PAGE under reducing conditions, blotting to nitrocellulose and detection with either streptavidin-HRP or by ligand blot using proMMP-9 as a probe.

FIGURE 9

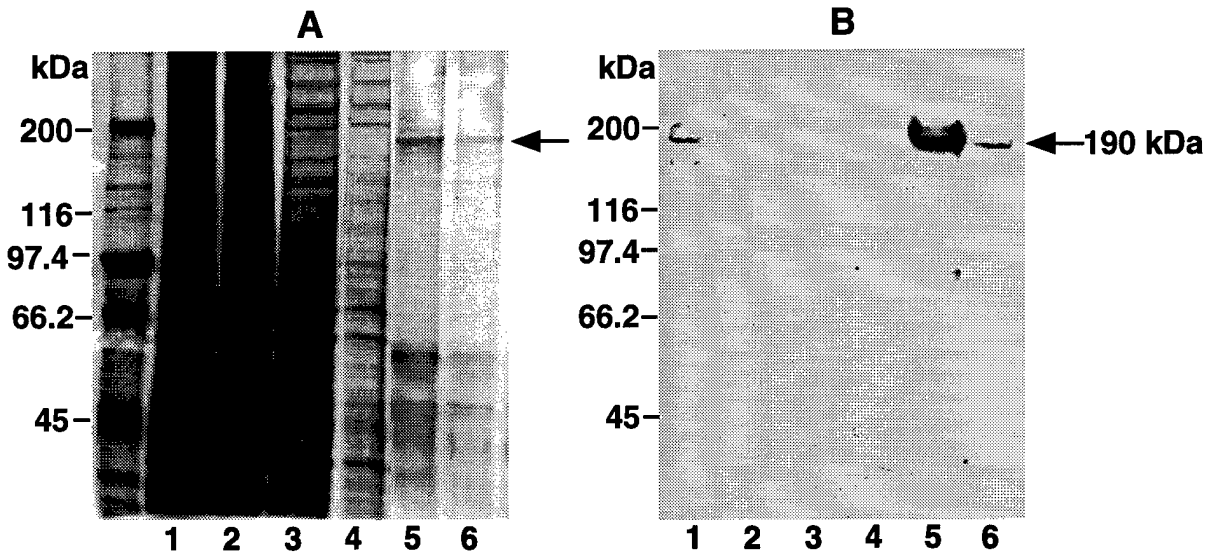

Figure 9. Affinity Purification of the 190-kDa proMMP-9 Binding Protein. A lysate (132.5 mg) of MCF10A cells was subjected to affinity purification using Affi-Gel 10-MMP-9 matrix as described under "Experimental Methods." The collected fractions were subjected to 4-12% SDS-PAGE under reducing conditions and detection by either silver staining (A) or ligand blot analysis (B). Lane 1, lysate before load (26.5 μg); lane 2, flow through (25 μg); lane 3, wash fraction 1 (17 μg); lane 4, wash fraction 2 (2.5 μg); lane 5, eluate fraction 1 (0.67 μg) and lane 6, eluate fraction 2 (>0.05 μg). The black arrows show the 190-kDa protein.

FIGURE 10

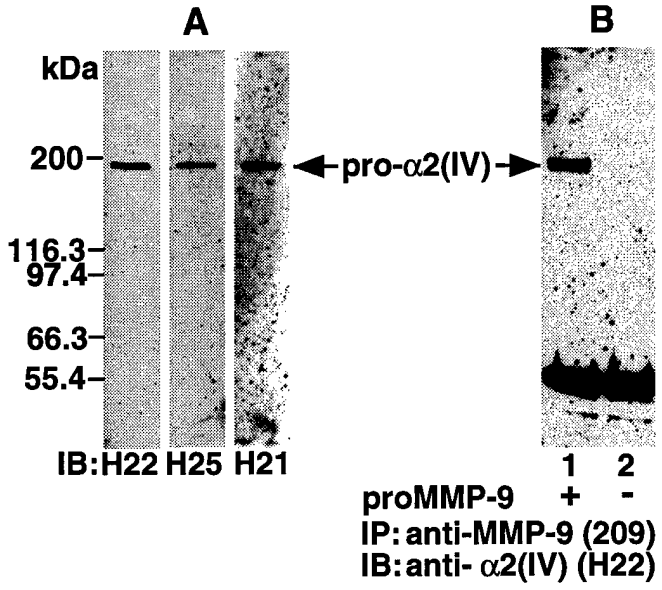

Figure 10. Immunoblot analysis of 190-kDa Protein with Chain-Specfic Antibodies. (**A**) Affinity-purified190-kDa protein (1 μg)/lane was subjected to SDS-PAGE under reducing conditions followed by immunoblot analysis with three different antibodies to the human pro-α2(IV) chain, H22, H25 and H21. (**B**) MCF10A lysates (1 ml) were incubated with (+) or without (-) 5 nM of recombinant proMMP-9 and then immunoprecipitated with anti-MMP-9 antibodies. The immunoprecipitates were resolved by SDS-PAGE under reducing conditions followed by immunoblot analysis with H22 antibodies.

FIGURE 11

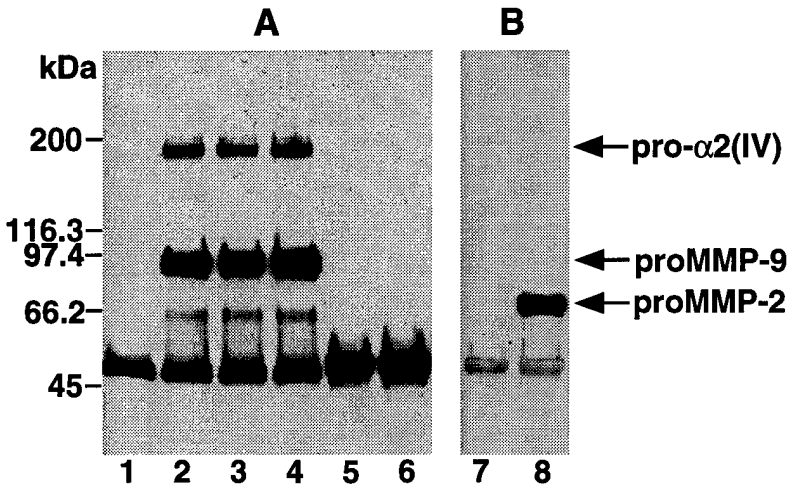

Figure 11. Co-immunopreciptation of pro-a2(IV) with proMMP-9 and proMMP-2. Affinity purified pro-2(IV) (0.5 ml) was incubated (1h, 4^0 C) with either proMMP-9 (5 nM, lanes 2-4) alone (lane 2) or together with proMMP-2 (lane 3, 5 nM; lane 4, 25 nM) or with proMMP-2 alone (5 nM, lanes 6 and 8) or without enzymes (lanes 1 and 5). The mixtures were then immunoprecipitated with either anti -MMP-9 (CA-209) antibodies (lanes 1-4) or with anti-MMP-2 (CA-801 and CA-805) antibodies (lanes 5-8) as described in the "Experimental Methods" section. The immunoprecipitates were resolved by SDS-PAGE under reducing conditions, transfer to nitrocellulose paper, and the blot subjected to ligand blot with MMP-9 as a probe (A) or immunoblot analysis (B) with a rabbit polyclonal antibody to MMP-2.

FIGURE 12

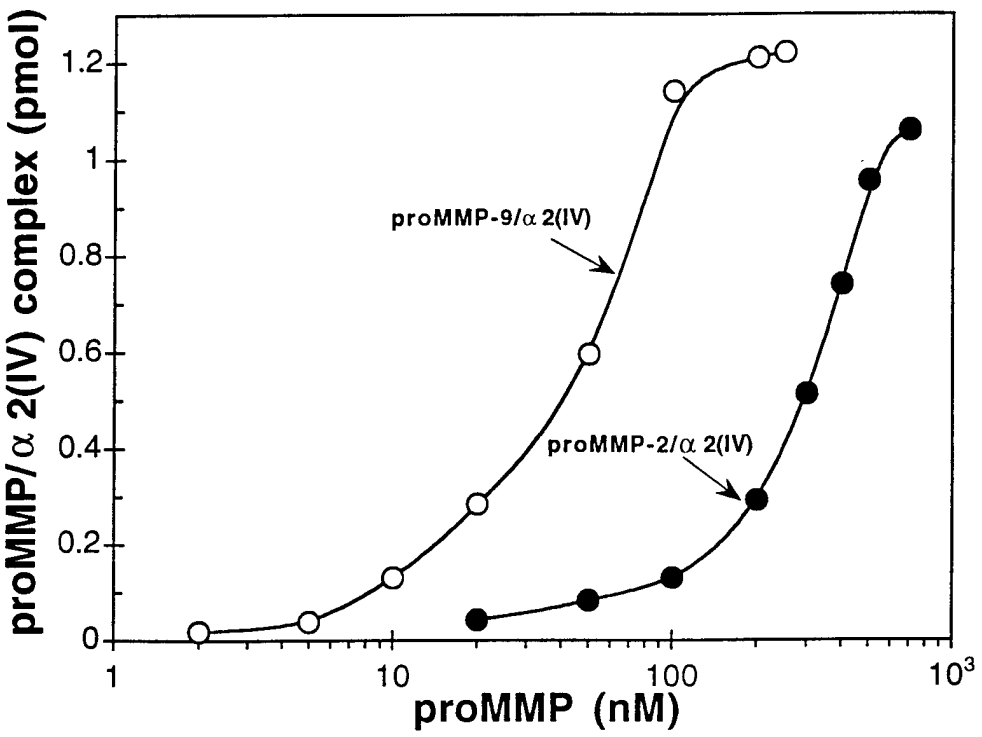

Figure 12. Complex Formation of proMMP-9 and proMMP-2 with pro-α2(IV) and Affinity Determination. Affinity purified pro-α2(IV) (180 ng) were allowed to complex with increasing concentrations of either ^{35}S-proMMP-9 (2-250 nM) or ^{35}S-proMMP-2 (20 to 750 nM) in a final volume of 100 µl. The complexes were then subjected to Superose 6 gel filtration chromatography. The amount of proMMP-9 and proMMP-2 complexed with pro-α2(IV) and the equilibrium binding constant were determined as described under "Experimental Methods".

FIGURE 13

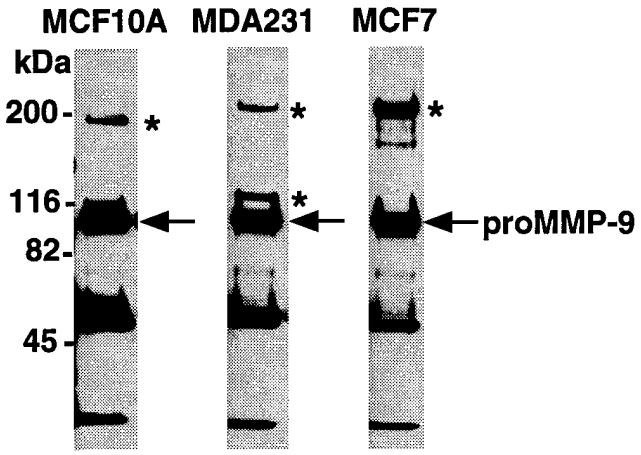

Figure 13. Expression of proMMP-9 Binding Proteins in Breast Epithelial Cells. Lysates (1 ml) of MCF10A (non-malignant), MDA-MB-231 (metastatic) and MCF7 (non-metastatic) were incubated with 5 nM of proMMP-9. The samples were then immunoprecipitated with anti-MMP-9 antibodies. The immunoprecipiates were resolved by SDS-PAGE under reducing conditions followed by ligand blot analysis using proMMP-9 as a probe as described in the "Experimental Methods" section. Note the presence of the pro-α2(IV) in MCF10A cells and a ~200-kDa protein in both MDA-MB-231 and MCF7 cells (arterisks). MDA-MB-231 cells also show presence of 120-kDa protein (arterisk).

FIGURE 14

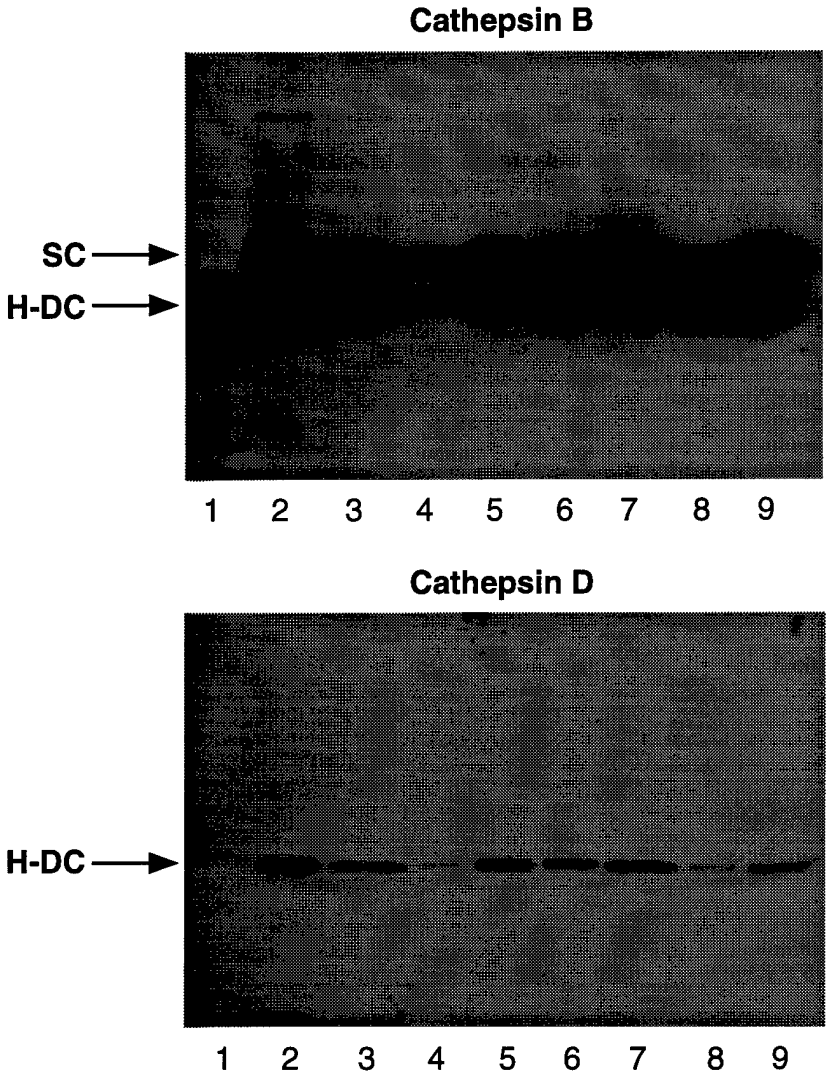

Figure 14. Immunoblot Analysis of Cathepsin B and D Expression in Primary Breast Fibroblasts. WS12B (lanes 2 and 3), WS12B virally transformed (lanes 4 and 5), WS12T (lanes 6 and 7) and WS12T virally transformed (lanes 8 and 9) were grown for 3 days in either uncoated (lanes 2,4,6, and 8) or collagen I coated (lanes 3,5,7, and 9) dishes as described in "Experimental Method". Cells were lysed and equal protein amounts of lysates were resolved by SDS-PAGE under reducing conditions followed by immunoblot analysis using a polyclonal antibody to human cathepsin B or to human cathepsin D. SC: shows the single chain and H-DC shows the heavy chain of double chain of either cathepsin B or D.

FIGURE 15

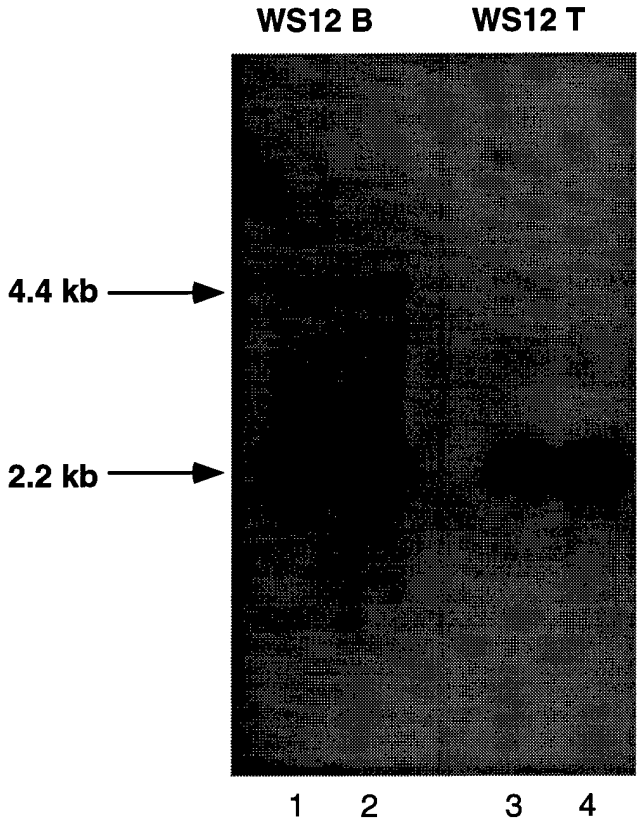

Figure 15. Northern Blot Analyses of Cathepsin B in Primary Breast Fibroblasts. WS12B and WS12T (both at passage 7) were cultured on either plastic (lane 1 and 3) or a collagen I gel (lanes 2 and 4). Total RNA (5 μg each)was then isolated as described in the "Experimental Methods" section, electrophoresed, blotted and hybridized with a human cathepsin B cDNA probe that detect two transcripts of 2.2 and 4.4 kb.

Appendix 1

[CANCER RESEARCH 57, 0000–0000, August 1, 1997]

Phorbol Ester-induced Cell Surface Association of Matrix Metalloproteinase-9 in Human MCF10A Breast Epithelial Cells[1]

Marta Toth, David C. Gervasi, and Rafael Fridman[2]

Department of Pathology and Karmanos Cancer Institute, Wayne State University, Detroit, Michigan 48201

ABSTRACT

Cell surface association of extracellular matrix (ECM)-degrading enzymes has been suggested to facilitate proteolysis of ECM in areas of cell-matrix contacts and to be crucial for the process of tumor cell invasion. Matrix metalloproteinase-9 (MMP-9) is a member of the MMP family of endopeptidases that has been shown to play a critical role in hydrolysis of ECM components and has been localized on the surface of tumor cells. However, the nature of the cell surface association of MMP-9 is unknown. Here, we report the cell surface association of MMP-9 in human breast epithelial MCF10A cells treated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Surface biotinylation and immunoprecipitation with anti-MMP-9 antibodies revealed the presence of two MMP-9 forms (M_r 92,000 and 85,000) on the surface of TPA-treated MCF10A cells, whereas in the media, only the M_r 92,000 form was detected, mostly in complex with TIMP-1, a specific MMP-9 inhibitor. The MMP-9 forms were also found in purified plasma membranes of TPA-treated cells. In contrast, the plasma membranes contained little or no TIMP-1. The surface-bound MMP-9 forms were recognized by an antibody to the NH_2-terminal prodomain, indicating that both represent latent enzymes. Pulse-chase analysis and endoglycosidase H digestion of surface-biotinylated MMP-9 forms demonstrated that the M_r 85,000 species was endoglycosidase H sensitive, suggesting targeting of the precursor form of MMP-9 to the cell surface. These studies demonstrate a specific cell surface association of MMP-9 in response to TPA that may help to localize TIMP-1-free enzyme on the surface of breast epithelial cells.

INTRODUCTION

The turnover of ECM[3] occurs primarily at areas of cell-matrix contacts and is carried out by proteinases intimately intertwined with the cell surface. Several families of ECM-degrading proteinases have been shown to be associated with the cell surface, including MMPs (1–3), plasminogen activator (4), and cathepsins B and D (5). Cell surface association of ECM-degrading enzymes allows for localized and controlled proteolysis of ECM components and prevents complete matrix dissolution, which would otherwise adversely influence the adhesive and migratory activities of cells and lead to extensive and irreparable tissue damage. It also allows cells to strictly regulate the initiation, inhibition, and termination of proteolytic activity by concentrating specific receptors, enzymes, and inhibitors in discrete areas of the cell surface. The regulation and function of cell surface-bound proteinases and their importance for localized degradation of ECM have been very well demonstrated with the plasminogen/plasmin system, in which cells developed a complex molecular system of proteases, protease receptors, and specific inhibitors that interact with each other to regulate proteolytic activity on the cell surface (4). In recent years, new studies provided evidence of the cell surface association and regulation of several members of the MMP family, including gelatinases (6–11) and membrane-type MMPs (3). The gelatinases, MMP-2 and MMP-9, have been shown to play a central role in the degradation of ECM during tissue remodeling, angiogenesis, arthritis, and tumor metastasis, and their expression is elevated in many human cancers (12, 13). The early studies of Zucker *et al.* (14–17) provided the first evidence of MMP-2 and MMP-9 activities on the PMs of tumor cells. Later studies showed the association of MMP-2 with the surface of transformed cells (8, 18). We have shown a pericellular localization of both MMP-2 and MMP-9 in the epithelium of fibrocystic disease of the breast and in the carcinoma cells of breast tumors (19). Others have shown the localization of MMP-9 at areas of tumor-basement membrane contacts in skin tumors (20). The association of the gelatinases with the cell surface may be an important factor in the regulation of proteolytic activity and may allow these enzymes to specifically degrade ECM proteins at areas of cell-matrix contacts and also to target surface proteins as shown with β-amyloid (21, 22), galectin-3 (23), and FGFR-1 (24).

Much information has been recently gained on the cell surface association of MMP-2, yet little is known about the interaction of MMP-9 with the cell surface. MMP-9 is produced by a variety of normal and tumor cells, including mesenchymal (25–27), epithelial (28, 29), endothelial (30), and inflammatory cells (25), and has been shown to play a role in many physiological and pathological processes, including trophoblast implantation (31), inflammation (32), bone resorption (33), arthritis (34), and cancer metastasis (35). Structurally, MMP-9 bears significant sequence similarities with MMP-2 but it also differs in substantial structural and regulatory elements (1, 12, 13). In addition to the classical domains of other MMP family members, both MMP-9 and MMP-2 contain a gelatin-binding domain that is homologous to the type II module present in fibronectin (1, 12, 13). MMP-9 possesses an additional 54-amino acid proline-rich region that is similar to the α2(V) chain of collagen V that is absent from MMP-2 (25), the function of which is unknown. MMP-9, unlike MMP-2, is heavily glycosylated due to the presence of three potential *N*-linked glycosylation sites (Asn_{19}, Asn_{101}, and Asn_{108}; $MMP\text{-}9_{688}$ notation) and several less defined *O*-linked glycosylation sites (25), but the role of the oligosaccharide moieties on MMP-9 function remains unknown. Like latent MMP-2 with TIMP-2, latent MMP-9 forms a noncovalent complex with TIMP-1 (25). The binding of TIMP-1 is mediated by the COOH-terminal domain of the proenzyme form and alters enzyme activation (36–39).

In contrast to MMP-2, which is usually constitutively expressed in many cultured cells, MMP-9 expression can be induced by TPA (40), growth factors (41, 42), cytokines (43), and by yet undefined factors in *in vitro* models of tumor-stroma interactions (35, 44). In many instances, as is the case after TPA treatment, induction of MMP-9 expression and secretion is accompanied by the simultaneous induction and secretion of TIMP-1, which binds to the secreted proenzyme form (25, 40). However, TPA treatment does not result in MMP-9 activation (7, 9, 25, 40, 45). In contrast, exposure to TPA induces the activation of latent MMP-2 by MT1-MMP (7, 9). While investigating the PM-dependent activation of MMP-2, we tested PMs isolated from a nonmalignant immortalized breast epithelial cell line, MCF10A (46)

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[2] To whom requests for reprints should be addressed, at the Department of Pathology, Wayne State University, 540 East Canfield Avenue, Detroit, MI 48201. Phone: (313) 577-1218; Fax: (313) 577-8180; E-mail: rfridman@med.wayne.edu.

[3] The abbreviations used are: ECM, extracellular matrix; MMP, matrix metalloproteinase; PMSF, phenylmethylsulfonyl fluoride; APMA, *p*-aminophenyl-mercuric acetate; ECL, enhanced chemiluminescence; HRP, horseradish peroxidase; endo-H, endoglycosidase H; PM, plasma membrane; PMCA, PM Ca^{2+} ATPase; TBS, Tris-buffered saline.

and found MMP-9 after exposing the cells to TPA. Here, we examined in detail the cell surface association of MMP-9 in MCF10A cells after TPA treatment.

MATERIALS AND METHODS

Cells. Human immortalized MCF10A breast epithelial cells, originally isolated from a patient with fibrocystic disease of the breast (46), were kindly provided by Dr. Bonnie Sloane (Wayne State University). The cells were grown in DMEM/F-12 (1:1) medium supplemented with 5% horse serum, 10 μg/ml insulin, 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml Fungizone, 0.5 μg/ml hydrocortisone, and 20 ng/ml epidermal growth factor. All tissue culture reagents were purchased from Life Technologies, Inc. (Grand Island, NY).

Reagents and Antibodies. TPA, aprotinin, leupeptin, pepstatin A, PMSF, APMA, tRNA, Triton X-114, and gelatin-agarose beads were all purchased from Sigma Chemical Co. (St. Louis, MO). Sulfo-NHS-biotin and the BCA protein determination kit were purchased from Pierce Chemical Co. (Rockford, IL). The ECL detection system, HRP-conjugated secondary antibodies, streptavidin-HRP, and [^{14}C]-labeled molecular weight standards were purchased from Amersham Corp. (Arlington Heights, IL). Percoll and Fast-flow protein G-Sepharose beads were purchased from Pharmacia (Piscataway, NJ). Label Express [^{35}S]methionine was purchased from NEN, (Wilmington, DE). Endo-H (25 units/ml) was purchased from Glyko (Novato, CA). BA-S 85 nitrocellulose membrane was purchased from Schleicher & Schuell (Keene, NH). Monoclonal antibodies to TIMP-1 (Ab-1) were purchased from Calbiochem Oncogene Research Products (Cambridge, MA). An anti-MMP-9 rabbit polyclonal antibody (pAB109) raised against a synthetic peptide (APRQRQSTLVLTPGDLRT) from the NH_2-terminal domain of human latent MMP-9 was a generous gift from Dr. Stetler-Stevenson (National Cancer Institute, NIH). The production and characterization of the monoclonal antibody CA-209 against human MMP-9 and T2–101 against human TIMP-2 were described previously (19). A rabbit polyclonal antibody to human TIMP-1 was a generous gift from Dr. B. Chua (Wayne State University). A rabbit polyclonal antibody raised against a synthetic peptide (RFNEELRAVDSEYPNIK) derived from the amino acid sequence of human MT1-MMP (3, 47) was produced by and purchased from Genetics Research, Inc. (Huntsville, AL). Monoclonal antibodies to PMCA were purchased from Affinity Bioreagents, Inc. (Golden, CO).

Expression, Purification, and Activation of Human Recombinant MMP-9. Human recombinant MMP-9 was produced in a recombinant vaccinia-mammalian cell expression system and purified to homogeneity by gelatin-agarose chromatography, as described previously (48). The protein concentration of MMP-9 was determined by its molar extinction coefficient of 114,360 $M^{-1}cm^{-1}$ (49). To obtain a mixture of latent (M_r 92,000) and active (M_r 82,000) MMP-9 forms, the latent MMP-9 was partially activated with 1 mM APMA for 2 h at 37°C (45). To isolate the M_r 82,000 species, 10 μg of latent MMP-9 were incubated with 200 ng of a recombinant catalytic domain of stromelysin 1 (a gift from Dr. Paul Cannon) for 2 h at 37°C (50) and then subjected to gelatin-agarose chromatography to remove stromelysin 1.

Gelatin Zymography. Gelatin zymography of conditioned media and PM fractions was performed using 10% Tris-glycine SDS-polyacrylamide gels containing 0.1% gelatin (45). Briefly, samples were mixed with Laemmli sample buffer without reducing agents and without heating and then subjected to SDS-PAGE. The gels were then incubated (30 min at 22°C) in renaturating buffer (2.5% Triton X-100 in H_2O), rinsed in distilled H_2O, and equilibrated for an additional 30 min in developing buffer (50 mM Tris buffer, pH 8.0, 200 mM NaCl, 5 mM $CaCl_2$, and 0.02% Brij-35) followed by an incubation (16 h at 37°C) in fresh developing buffer. The gels were then stained with 0.5% Coomassie Blue R250 in a solution of 10% methanol and 5% acetic acid and then destained in 10% methanol and 5% acetic acid.

Immunoblot Analysis. Immunoblot analyses were carried out with samples of conditioned media and purified PM fractions. Samples (200 μl) of serum-free conditioned medium were concentrated by the addition of 20% trichloroacetic acid and 25 μg/ml of carrier tRNA (final concentrations). The mixtures were incubated for 1 h at 4°C and centrifuged (15 min at 13,000 × *g*), and the pellet was resuspended in Laemmli sample buffer and boiled. In some experiments, the serum-free conditioned medium of TPA-treated MCF10A cells was subjected to gelatin-agarose chromatography to purify MMP-9 and the MMP-9/TIMP-1 complex as described (48). Purified PMs were resuspended in sample buffer and boiled. The samples were subjected to SDS-PAGE followed by transfer to a BA-S 85 nitrocellulose membrane. The blots were blocked overnight at 4°C with 3% BSA and 3% nonfat dry milk in 100 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.02% NaN_3 (blotto) and incubated for 1 h with the corresponding primary antibody in 20 mM Tris-HCl, pH 7.5, containing 137 mM NaCl and 0.1% Tween 20 (T-TBS). After being washed three times with T-TBS, the blots were incubated with the appropriate HRP-conjugated secondary antibody. Detection of the immune complexes was performed using the ECL system according to the manufacturer's instructions.

Cell Surface Biotinylation. MCF10A cells in 150-mm dishes were treated with 100 nM TPA (from a 2 mM stock solution in ethanol) for 2 h at 37°C. Control cells received vehicle alone (final concentration, 0.005% ethanol). After being washed four times with serum-free medium, the cells were incubated in serum free-medium for 18 h. The cells were then washed three times with cold PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS-CM). Biotinylation of cell surface proteins was carried out by incubating the cells with 0.5 mg/ml of the water-soluble, cell-impermeable, biotin analogue sulfo-NHS-biotin for 30 min at 4°C in PBS-CM. The biotinylation reaction was quenched with 50 mM NH_4Cl in PBS-CM followed by two washes with cold PBS. The cells were then ready for Triton X-114 phase separation.

Triton X-114 Extraction and Immunoprecipitations. Biotinylated and nonbiotinylated TPA-treated and control MCF10A cells were solubilized with 1.5% Triton X-114 in TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 2 mM benzamidine, and 5 mM EDTA. The extracts were centrifuged (15 min at 14,000 × *g* at 4°C), and the supernatants were warmed (2 min at 37°C) and centrifuged (14,000 × *g* at 22°C) to separate the lower, detergent and upper, aqueous phases. The aqueous phase was then incubated (1 h at 4°C) with gelatin-agarose beads followed by a brief centrifugation. The beads were then washed three times with cold TBS containing 5 mM $CaCl_2$ and 0.02% Brij-35, and the bound proteins were eluted in the same buffer containing 10% DMSO. After a brief centrifugation, the supernatant containing the eluted proteins was diluted (5-fold) with immunoprecipitation buffer (50 mM Tris buffer, pH 7.5, 150 mM NaCl, 0.05% Brij-35, and 1 mM PMSF). The samples were then incubated with various anti-MMP-9 antibodies or mouse or rabbit IgG, such as controls and protein G-Sepharose beads. The immunoprecipitates were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. After being blocked with blotto and washed with T-TBS, as described above, detection of the biotinylated proteins was accomplished using a HRP-conjugated streptavidin and the ECL detection system. The specificity of the detection was determined in nonbiotinylated cells extracted and immunoprecipitated as described above.

Subcellular Fractionation. MCF10A cells were grown in roller bottles (20 per isolation) to 80% confluence and then treated for 12 h or not treated not with 100 nM TPA in serum-free medium. Control cells received vehicle alone. All of the following procedures were performed in the cold. The medium was aspirated and the cell monolayers were washed three times with cold PBS and scraped into PBS. After a 20-min centrifugation (1200 × *g*) at 4°C, the pellet was resuspended in 150 ml of 25 mM Tris-HCl, pH 7.5, containing 8.5% sucrose, 50 mM NaCl, 5 mM EDTA, 10 mM NEM, 10 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, and 1 mM PMSF. The suspension was homogenized in a Dounce homogenizer. The homogenate was centrifuged (10 min at 3000 × *g*) and the supernatant (postnuclear fraction) was collected and centrifuged (140,000 × *g*) in a SW28 rotor for 2 h at 4°C. The supernatant (cytosol) was collected, and the pellet (crude membranes) was resuspended in 10 ml of 25 mM Tris-HCl, pH 7.5, 50 mM NaCl buffer with protease inhibitors and applied to a discontinuous sucrose gradient (20/30/50/60%) in water. The samples were then centrifuged (140,000 × *g*) in a SW28 rotor for 2 h at 4°C, and the 30–50% interface was collected and resuspended in the same buffers as described above and centrifuged again for 2 h at 140,000 × *g*. The PM pellet was resuspended in the same buffer to a final concentration of 1.5–2.5 mg/ml. The enriched PM fraction was stored at −80°C or further purified in a Percoll gradient. To this end, 1 ml of PM (0.8 mg) was mixed with 9 ml of a solution of 33.3% Percoll in the same buffer. The mixture was then centrifuged (30,000 × *g*) in a Ti 70.1 rotor for 30 min. The purified PM appeared as a visible band at a density of 1.048 g/ml and was collected and pelleted by centrifugation (100,000 × *g* for 1 h). The enrichment of the preparation in PM

components was assessed by immunoblot analysis using antibodies to PMCA and MT1-MMP (3), two known PM proteins, and by measuring the activity of alkaline phosphodiesterase, a PM marker (51). Briefly, triplicate samples (20 μl) of either homogenate, postnuclear fraction, cytosol, or PM were incubated (18 h at 37°C) with 250 μl of substrate (10 mM TMP *p*-nitrophenil ester). After incubation, the reaction was quenched with 1 ml of 0.4 M sodium carbonate in 0.22 M glycine, pH 10. The absorbance of the samples was determined in a spectrophotometer at a wavelength of 405 nm. Enzyme activity was expressed as absorbance unit/mg protein. These studies showed both PMCA and MT1-MMP were present in the enriched PM fraction and a 15-fold increase in phosphodiesterase activity compared to the postnuclear fraction.

To determine the nature of the association of MMP-9 with the PM, a PM aliquot (25 μg of total protein) was resuspended in either PBS, 1 M NaCl, or 0.1 M Na_2CO_3, pH 11.5, and incubated for 30 min at 4°C. The samples were then centrifuged (1 h at 100,000 × *g*), and the supernatant and pellet were collected followed by the addition of Laemmli sample buffer. For phase separation with Triton X-114, an aliquot (25 μg of protein) of PM was incubated (15 min at 4°C) with Triton X-114 and centrifuged (15 min at 13,000 × *g* and 4°C), and the pellet was washed twice with PBS. The supernatant was transferred to a new tube and then phase separated by a 2-min incubation at 37°C followed by brief centrifugation at room temperature to generate the aqueous and the detergent phase. Then, the aqueous, detergent, and pellet fractions were each mixed with Laemmli sample buffer followed by immunoblot analysis.

Pulse-Chase Analysis. MCF10A cells were grown to 80% confluence in 60-mm dishes and then treated (12 h) with 100 nM TPA in serum-free medium. The medium was aspirated and the cell monolayer was washed twice with warm (37°C) PBS followed by incubation (45 min) with 1 ml/dish of starving medium (DMEM without methionine supplemented with 25 mM HEPES). The cells were then pulsed with 500 μCi/ml of [^{35}S]methionine in starving medium (0.6 ml/dish) for 15 min at 37°C. After the pulse, the dishes were placed on ice, the medium was aspirated, and the cells were washed twice gently with PBS before addition of 1 ml/dish of chase medium (DMEM with 10% fetal bovine serum and 4.8 mM methionine). At the end of the chase period (0–120 min at 37°C), the medium was collected, centrifuged (5 min at 12,000 × *g*), and transferred to a clean tube with the addition of harvest buffer (60 mM Tris-HCl, pH 7.5, containing 0.5% SDS, 2 mM EDTA, and 10 mM methionine, final concentrations). The samples were boiled (3 min), centrifuged, and transferred to a new tube with the addition of 5 mM iodoacetamide, 2.5% Triton X-100 and 20 μg/ml aprotinin (final concentrations). The cell monolayers were washed twice with D-PBS and lysed in 0.5 ml/dish of warm harvest buffer. The lysates were then subjected to five cycles of boiling and freezing followed by a brief centrifugation. The supernatants were collected into new tubes with the addition of 5 mM iodoacetamide, 2.5% Triton X-100, and 20 μg/ml aprotinin (final concentrations). For immunoprecipitations, the medium and lysate samples were incubated (16 h at 4°C) with 5 μg of CA-209 anti-MMP-9 antibodies followed by the addition of 30 μl of protein G-Sepharose beads for an additional 3-h incubation at 4°C. After the beads were recovered by a brief centrifugation, the supernatant was subjected to sequential rounds of immunoprecipitations with antibodies to TIMP-1, MT1-MMP, and TIMP-2. The recovered beads were washed (five times) with cold 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.1% NP-40, and 10% glycerol and resuspended in 15 μl of Laemmli sample buffer with DTT followed by boiling (5 min). Samples of media and lysates were resolved by 8–16% SDS-polyacrylamide gels. Detection of radiolabeled proteins was performed by autoradiography.

For coprecipitation of the MMP-9/TIMP-1 complex, an aliquot of the [^{35}S]-labeled medium from the 2-h chase period from the pulse-chase experiment was subjected to two consecutive rounds of immunoprecipitations with anti-MMP-9 and anti-TIMP-1 antibodies, respectively. To this end, the [^{35}S]-labeled medium, without boiling and without addition of harvest buffer, iodoacetamide, or Triton X-100, was incubated with anti-MMP-9 antibodies and protein G-Sepharose beads as described above. After the first centrifugation, the immunoprecipitates were processed as described above, and the supernatants were collected and subjected to another round of immunoprecipitation with anti-TIMP-1 antibodies using the same protocol. The immunoprecipitates were resolved by 8–16% SDS-PAGE under reducing conditions followed by autoradiography.

Endo-H Digestion. The immunoprecipitates with anti-MMP-9 from the biotinylated and pulse-chase samples were washed with TBS, centrifuged, and then resuspended in 25 μl of 100 mM sodium phosphate buffer, pH 5.5, containing 50 mM 2-mercaptoethanol and 0.1% SDS. The samples were then boiled (5 min) and cooled, followed by the addition of 2 μl of endo-H (25 units/ml). After incubation (2 h, 37°C), the samples were boiled in the presence of Laemmli sample buffer with reducing agents and subjected to SDS-PAGE followed by either immunoblot analysis (biotinylated samples) or autoradiography (pulse-chase samples).

RESULTS

Surface Association of MMP-9 but not of TIMP-1 in TPA-treated MCF10A Cells. Cultured MCF10A cells secrete low amounts of latent MMP-9 and TIMP-1. Treatment of the cells with 100 nM TPA for a period as short as 2 h induced the expression and secretion of high amounts of latent MMP-9 and TIMP-1, as determined by gelatin zymography (Fig. 1*A*) and by immunoblot analysis of TCA-precipitated medium (Fig. 1*B*). Exposure of cells to TPA for more than 2 h did not alter the level of MMP-9 and TIMP-1 expression in the medium. Gelatin affinity chromatography of medium from TPA-treated cells followed by immunoblotting with antibodies to MMP-9 and TIMP-1 (Fig. 1*C*) demonstrated that the enzyme copurified with TIMP-1 consistent with complex formation. To further examine the medium for the presence of the complex, an immunoprecipitation of MMP-9 and TIMP-1 was carried out from [^{35}S]-labeled medium of TPA-treated MCF10A cells as described in "Materials and Methods." As shown in Fig. 1*D*, the anti-MMP-9 antibodies precipitated MMP-9 in complex with TIMP-1 (*Lane 1*). Subsequent immunoprecipitation of the same medium with anti-TIMP-1 antibodies showed presence of TIMP-1 (*Lane 3*), suggesting that free inhibitor remained after removal of the MMP-9/TIMP-1 complex and that MCF10A cells secrete excess inhibitor over enzyme after treatment with TPA. The small amount of MMP-9 detected with TIMP-1 (Fig. 1*D*, *Lane 3*) was not enzyme coprecipitated with the inhibitor because it could be detected in the control precipitate without antibody (Fig. 1*D*, *Lane 4*) and represented MMP-9 remaining in the sample after the first immunoprecipitation.

To determine whether MMP-9 and TIMP-1 were associated with

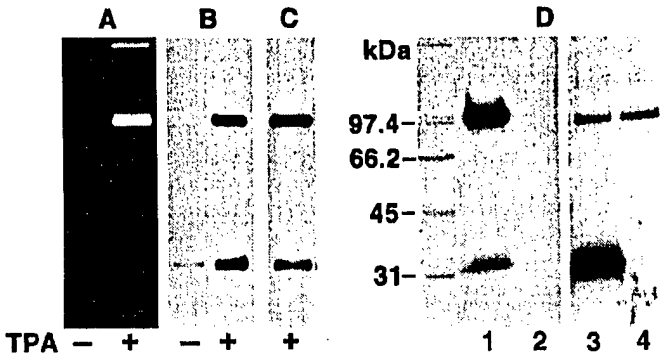

Fig. 1. Induction of MMP-9 and TIMP-1 expression in MCF10A cells by TPA. MCF10A cells were treated with 100 nM TPA (+) or vehicle alone (−) as described in "Materials and Methods." The medium was collected 18 h later and analyzed for MMP-9 expression by gelatin zymography (*A*) or immunoblot analysis (*B* and *C*). *B*, 200 μl of medium from untreated and TPA-treated MCF10A cells was TCA precipitated, resolved in a 8–16% SDS-PAGE under reducing conditions, blotted to nitrocellulose, and analyzed for MMP-9 and TIMP-1 expression using both anti-MMP-9 and anti-TIMP-1 antibodies and the ECL detection system. *C*, immunoblot analysis of MMP-9 and TIMP-1 expression in medium from TPA-treated cells subjected to gelatin-agarose chromatography as described in "Materials and Methods." The eluted samples were subjected to 8–16% SDS-PAGE under reducing conditions and immunoblot analysis as in *B*. *D*, [^{35}S]-labeled medium of TPA-treated cells was immunoprecipitated with a polyclonal anti-MMP-9 antibody (*Lane 1*), followed by a second round of immunoprecipitation with a polyclonal anti-TIMP-1 antibody (*Lane 3*). Control samples received no antibodies (*Lanes 2* and *4*). Immune complexes were resolved by 8–16% SDS-PAGE under reducing conditions followed by detection by autoradiography. ^{14}C-labeled molecular weight standards were used as reference.

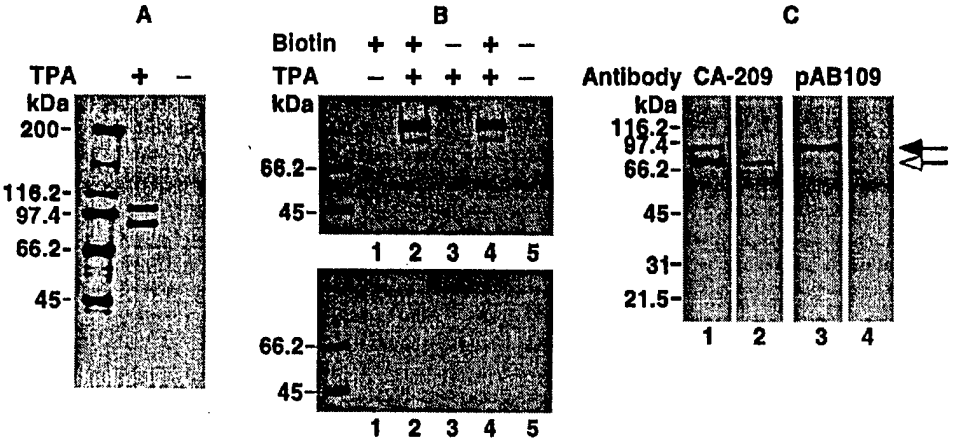

Fig. 2. Detection of MMP-9 in surface-biotinylated TPA-treated MCF10A cells. *A*, monolayers of MCF10A cells were treated (2 h) with 100 nM TPA (+) or vehicle alone (−) and then biotinylated with sulfo-NHS-biotin. After solubilization with Triton X-114, the aqueous phase was incubated with gelatin-agarose beads, and the eluted proteins were immunoprecipitated with anti-MMP-9 (CA-209) and protein G-Sepharose beads. The immunoprecipitates were resolved by 4–12% SDS-PAGE under reducing conditions, blotted to nitrocellulose paper, and developed with streptavidin-HRP and the ECL detection system. *B*, MCF10A cells treated with 100 nM TPA (+) or vehicle alone (−) were biotinylated (+) or not (−) and were processed as described in *A*. The samples were then immunoprecipitated with either CA-209 (*Lanes 1–3* and *5*, *top panel*) or pAB109 (*Lane 4*, *top panel*) or without antibodies (*bottom panel*) and subjected to 8–16% SDS-PAGE under reducing conditions, blotted to nitrocellulose paper, and developed with streptavidin-HRP and the ECL detection system. *C*, recombinant MMP-9 was either partially activated with 1 mM APMA (*Lanes 1* and *3*) to obtain a mixture of latent (M_r 92,000) and active (M_r 82,000) forms or activated to the M_r 82,000 form by stromelysin 1 (*Lanes 2* and *4*) as described in "Materials and Methods." The enzymes were subjected to 8–16% SDS-PAGE under reducing conditions and blotted to nitrocellulose paper. The blots were incubated with either pAB109 (against the NH_2-terminal domain) or monoclonal antibodies to MMP-9 (CA-209). Immune complexes were detected with the ECL detection system. *C*, *filled arrow*, latent MMP-9; *open arrow*, active form.

the cell surface, control and TPA-treated MCF10A cells were surface biotinylated and subjected to phase partitioning with Triton X-114. The resultant aqueous phase was incubated with gelatin-agarose beads, and the eluted proteins were immunoprecipitated with anti-MMP-9 antibodies. As shown in Fig. 2*A*, CA-209 antibodies precipitated a biotinylated M_r 92,000 protein from the extracts of TPA-treated MCF10A cells but not from the untreated cells consistent with cell surface localization of MMP-9. The CA-209 antibodies also precipitated a biotinylated protein with a M_r of approximately 85,000 from the TPA-treated cells (Fig. 2*A*). Although the M_r 85,000 form was consistently biotinylated, its intensity varied in different experiments. The amount and pattern of cell surface biotinylated MMP-9 were not affected by the duration of the TPA treatment (2–18 h). A biotinylated protein with the molecular weight of TIMP-1 (~31,000) could not be detected under these conditions (Fig. 2*A*). The lack of TIMP-1 coprecipitation with MMP-9 from the surface-biotinylated cells was not due to dissociation of the complex during phase separation with Triton X-114 because TIMP-1 could be readily coimmunoprecipitated with MMP-9 when culture medium or a purified recombinant MMP-9/TIMP-1 complex was subjected to the same extraction procedure (data not shown). Direct immunoprecipitation of TIMP-1 from lysates of surface-biotinylated MCF10A cells was unreliable due to the presence of multiple nonspecific proteins coprecipitating with the anti-TIMP-1 polyclonal antibodies (data not shown).

To determine whether the biotinylated M_r 85,000 form was activated MMP-9, we carried out an immunoprecipitation of surface-biotinylated cells with CA-209 antibodies and with a polyclonal antibody (pAB109) directed against the NH_2-terminal prodomain of MMP-9 (Fig. 2*B*). The results of this experiment showed that the immunoprecipitation pattern obtained with the pAB109 antibodies (Fig. 2*B*, *Lane 4*, *top panel*) was identical to that obtained with CA-209 (Fig. 2*B*, *Lane 2*, *top panel*), suggesting that the M_r 85,000 species also contains the complete NH_2-terminal prodomain. This finding and the fact that all of the procedures used to extract the cell surface associated MMP-9 were carried out in the presence of EDTA suggest that the M_r 85,000 species is a latent MMP-9 isoform. Detection of the biotinylated M_r 92,000 and 85,000 MMP-9 forms was specific because immunoprecipitations of either untreated, nonbiotinylated (Fig. 2*B*, *Lane 5*, *top panel*) or TPA-treated, nonbiotinylated (Fig. 2*B*, *Lane 3*, *top panel*) MCF10A cells did not produce signals. In addition, no positive signals were detected with samples subjected to the same protocols but without addition of antibodies (Fig. 2*B*, *bottom panel*). The ability of the two MMP-9 antibodies to recognize either latent or active MMP-9 was confirmed by immunoblot analysis, and CA-209 recognized both active and latent forms (Fig. 2*C*, *Lanes 1* and 2), whereas pAB109 reacted only with latent enzyme (Fig. 2*C*, *Lanes 3* and 4), as expected.

MMP-9 Is Present in the PM of TPA-treated MCF10A Cells. Because MMP-9 but not TIMP-1 was detected on the surface of TPA-treated MCF10A cells, we wished to determine whether MMP-9 could also be identified on isolated PM. As shown in the zymogram in Fig. 3*A*, the PM fractions contained M_r 92,000 and 85,000 gelatinolytic forms that were identified as MMP-9 by immunoblot analysis (Fig. 3, *B* and *D*). Both CA-209 and pAB109 antibodies recognized the MMP-9 forms as shown with the surface-biotinylated enzymes

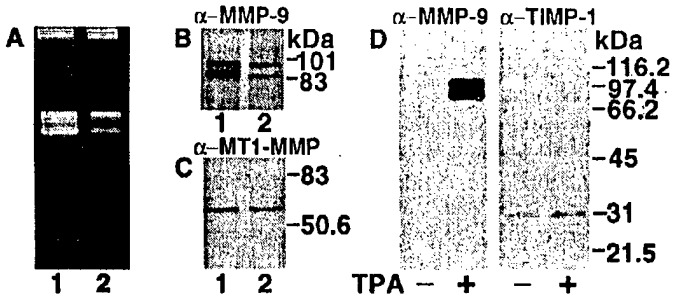

Fig. 3. Localization of MMP-9 in enriched-PM fractions of MCF10A cells. Gelatin-zymography (*A*) and immunoblot analysis (*B–D*) of PM isolated from MCF10A cells treated with TPA (*A–C* and + in *D*) or vehicle alone (*D*, −) by sucrose gradient (*A–C*, *Lane 1* and *D*) and by Percoll gradient (*A–C*, *Lane 2*). PM fractions (10 μg/lane) were subjected to gelatin-zymography as described in "Materials and Methods." For immunoblot analysis, enriched-PM fractions (*B* and *C*, 10 μg/lane; *D*, 45 μg lane) were subjected to 8–16% SDS-PAGE under reducing conditions and blotted to nitrocellulose paper for detection with either anti-MMP-9 (CA-209; *B* and *D*), anti-MT1-MMP (*C*), or anti-TIMP-1 (*D*) antibodies. *D*, note that the exposure time of the film with the anti-TIMP-1 antibodies was 15 min, whereas that of the anti-MMP-9 antibodies was 1 min.

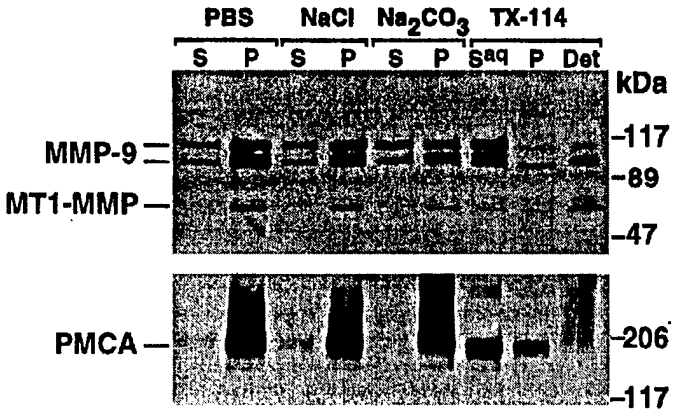

Fig. 4. Analysis of the PM association of MMP-9. Enriched PM fractions (25 μg) from TPA-treated MCF10A cells were treated with either PBS, 1 M NaCl, or 0.1 M Na_2CO_3 or subjected to phase separation with Triton X-114. After PBS, 1 M NaCl, and 0.1 M Na_2CO_3 treatments, the PM suspensions were centrifuged to obtain the supernatant (*S*) and pellet (*P*). Triton X-114 separation of the PM into the aqueous (S^{aq}) and detergent (*Det*) phases and insoluble pellet (*P*) was achieved as described in "Materials and Methods." The samples were resolved by SDS-PAGE under reducing conditions followed by immunoblot analysis with anti-MMP-9, anti-MT1-MMP, and anti-PMCA antibodies.

(Fig. 2*B*), suggesting that both MMP-9 forms on the PM represent latent enzymes. In some occasions, the M_r 85,000 form appeared as a doublet; however, this was inconsistent. The zymogram also showed a high molecular weight (~200,000) gelatinolytic band, possibly representing the MMP-9 homodimer. No differences were observed in PM preparations that were further purified by Percoll gradient. As a control, the PM fractions were subjected to immunoblot analysis for expression of MT1-MMP (Fig. 3*C*). Association of MMP-9 with the PM was only shown after TPA-treatment (Fig. 3*D*). In contrast, TIMP-1 was barely detected in the PM of untreated and TPA-treated cells (Fig. 3*D*), in agreement with the lack of detection of TIMP-1 in the surface of the biotinylated cells.

The nature of the association of MMP-9 to the PM was examined by subjecting the PM fraction to various treatments. After treatment, the PM suspension was centrifuged, and the pellet and the supernatant were examined for the presence of MMP-9 by immunoblot analysis. As a control, these fractions were also examined for the presence of PMCA and MT1-MMP. As shown in Fig. 4, extensive washes of the PM with PBS or treatment with 1 M NaCl released only a low amount of MMP-9 into the supernatant, whereas most of the enzyme remained bound to the pellet after equal proportions of each sample were analyzed. Treatment of the PM with 0.1 M Na_2CO_3, pH 11.5, showed an apparently equal distribution of MMP-9 between the supernatant and the pellet. Neither PMCA nor MT1-MMP was released from the PM by these treatments, as expected. Phase partitioning of the PM preparation with Triton X-114 showed that most of the MMP-9 forms were released into the aqueous phase (Fig. 4, S^{aq}). However, a small amount, containing mostly the M_r 85,000 form, remained in the detergent phase (*Det*) and in the insoluble pellet (*P*). Fig. 4 also shows the effects of these treatments on the distribution of MT1-MMP and PMCA, which demonstrated that these two proteins behave like integral PM proteins with the exception of the reported anomalous distribution of PMCA into the aqueous phase after phase separation with Triton X-114, as expected. Taken together, these results suggest that PM-associated MMP-9 behaves like a peripheral membrane protein. However, the low molecular weight form of MMP-9 remained strongly bound to the PM and could not be extracted by any of these treatments.

Pulse-Chase Analysis of MMP-9 Synthesis in TPA-treated MCF10A Cells. Because two MMP-9 latent forms were biotinylated on the cell surface and bound to the PM, whereas only the M_r 92,000 form was found in the culture medium, we investigated the biosynthetic pathway of MMP-9 in TPA-treated MCF10A cells by pulse-chase analysis. The synthesis of MMP-9 was compared with that of TIMP-1, TIMP-2, and MT1-MMP (Fig. 5). After a 15-min pulse, the intracellular (cellular fraction) and extracellular (medium) fractions were harvested at various times (0–120 min) and immunoprecipitated with the appropriate antibody. As shown in Fig. 5, a protein of M_r 83,000–85,000, probably representing the precursor form of MMP-9, was detected at the 0-min chase in the cell lysate. After a 15-min chase, the M_r 85,000 MMP-9 precursor form was gradually converted to the mature enzyme (M_r 92,000), consistent with processing of complex oligosaccharide chains. Both forms were clearly detectable intracellularly even after a 120-min chase. Analysis of the medium (Fig. 5, *extracellular panels*) from the same experiment demonstrated a gradual secretion of the M_r 92,000 proenzyme starting after the 30-min chase, in agreement with the time (15 min) of processing of the precursor form into the mature proenzyme (92,000). In the medium, the presence of low molecular weight forms of the enzyme could not be detected.

The biosynthetic pathway of MMP-9 in TPA-treated cells was compared to that of TIMP-1, TIMP-2, and MT1-MMP. The TIMPs, like MMP-9, are secreted proteins, whereas MT1-MMP is membrane bound. As shown in Fig. 5, the TIMP-1 precursor form was readily detected at time 0 and was gradually glycosylated and secreted from the cells after a 60-min chase. After 90 min, TIMP-1 was barely detected intracellularly. It should be noted that the denaturing conditions of the pulse-chase protocol do not allow for determination of complex formation in the intracellular and extracellular compartment. TPA-treated MCF10A cells also produce TIMP-2, which was rapidly synthesized and secreted. However, in contrast to TIMP-1, a fraction of TIMP-2 remained in the intracellular compartment even after a 6-h chase (data not shown). Pulse-chase analysis of the biosynthetic pathway of MT1-MMP showed that 15 min after synthesis, the proenzyme form (M_r ~65,000–63,000) was converted to a lower molecular weight form (M_r ~60,000), consistent with intracellular processing. This form was not further processed and remained cell associated. Taken together, these studies demonstrated a unique processing of MMP-9 compared to that of TIMP-1, in which both MMP-9 forms remained associated with the cell compartment, whereas only the mature form was secreted.

Endo-H Digestion of Intracellular and Cell Surface MMP-9. Because MMP-9 is a glycosylated enzyme (25), we examined the glycosylation pattern of the cell surface-associated and intracellular forms of MMP-9 by endo-H digestion. This enzyme specifically cleaves oligosaccharides of the high-mannose, hybrid type and helps to distinguish complex carbohydrates, usually present in the mature form of secreted glycoproteins, from high-mannose oligosaccharides. We postulated that the low molecular weight form of MMP-9 on the cell surface and PM fraction may be a differentially glycosylated MMP-9 latent form. We wished to determine the similarities and differences in glycosylation between the cell surface M_r 85,000 form detected after biotinylation and the intracellular M_r 85,000 precursor form, detected in the pulse-chase experiment. To this end, the immunoprecipitates of the surface-biotinylated cells (cell surface) and of the pulse-chase experiment (intracellular) collected after 0- and 30-min chase periods were subjected to endo-H treatment. Fig. 6 shows that the intracellular M_r 85,000 precursor form was endo-H-sensitive (Fig. 6, *Lanes 2* and *4*), consistent with the presence of *N*-linked high-mannose oligosaccharides, whereas the intracellular M_r 92,000 form was resistant (Fig. 6, *Lane 4*), consistent with the addition of complex carbohydrates to the mature M_r 92,000 form in the Golgi complex. Endo-H digestion of the surface-biotinylated MMP-9 forms revealed that the M_r 85,000 enzyme was endo-H sensitive, whereas the M_r

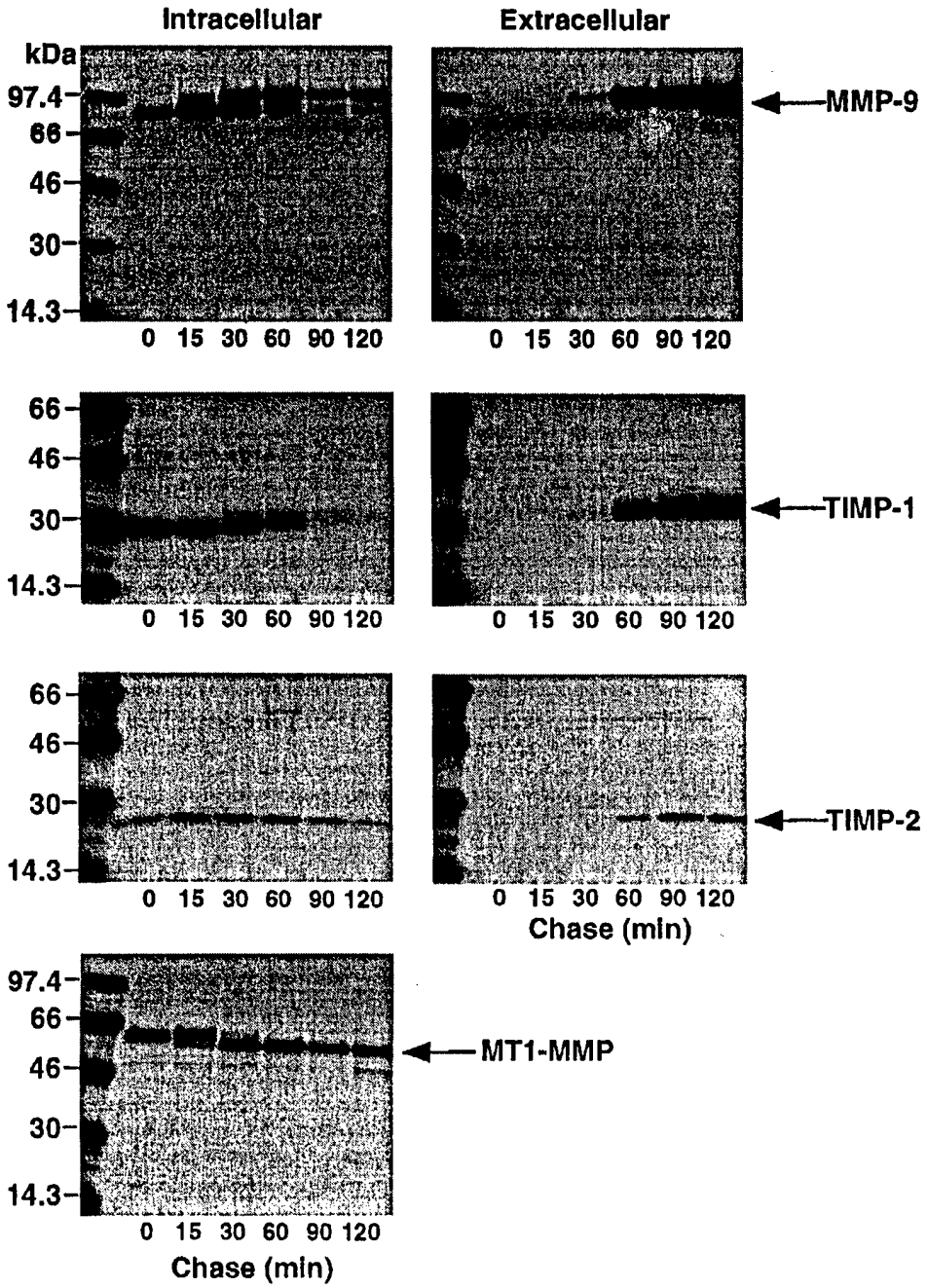

Fig. 5. Pulse-chase analysis of MMP-9, TIMP-1, TIMP-2, and MT1-MMP synthesis in TPA-treated MCF10A cells. Cells were treated with 100 nM TPA and subjected to pulse-chase analysis as described in "Materials and Methods." At the end of the chase period (0–120 min), the cells (intracellular) and the medium (extracellular) were subjected to consecutive rounds of immunoprecipitations with antibodies to MMP-9, TIMP-1, MT1-MMP, and TIMP-2. The immunoprecipitates were subjected to 8–16% SDS-PAGE analysis under reducing conditions followed by autoradiography. ^{14}C-labeled molecular weight standards were used as reference.

92,000 form was resistant (Fig. 6, *Lane 6*). Consistently, secreted MMP-9 was also endo-H resistant (data not shown). Taken together, these results show that the cell surface-associated M_r 85,000 MMP-9 latent form possesses a glycosylation pattern that is similar to that of the intracellular M_r 85,000 precursor form. Thus, the cell surface-associated M_r 85,000 form probably represents a unique processing and targeting to the cell surface of the precursor form of MMP-9 in TPA-treated MCF10A cells.

DISCUSSION

In this study, we present evidence on the cell surface association of MMP-9 in human MCF10A breast epithelial cells treated with TPA. This was demonstrated by immunoprecipitation with anti-MMP-9 antibodies of surface-biotinylated MCF10A cells that revealed the presence of two MMP-9 forms with molecular weights of approximately 92,000 and 85,000 on the cell surface. Immunoprecipitation of the surface-biotinylated cells with an antibody directed against the NH_2-terminal propeptide of MMP-9 demonstrated that both enzymes contain the complete propeptide domain and consequently are in the latent form. Thus, in TPA-treated MCF10A cells, appearance of the M_r 85,000 form on the cell surface is not the result of proenzyme activation. Consistent with these results, the two MMP-9 forms were also identified in an enriched PM fraction of TPA-treated cells. To elucidate the nature of the M_r 85,000 form on the cell surface, we carried out pulse-chase analysis experiments and endo-H digestion of precursor and mature forms of MMP-9. These studies showed a gradual intracellular processing of the precursor form (M_r 85,000) of MMP-9 into the mature enzyme. Whereas the latter were secreted into the medium, the M_r 85,000 form remained in the cellular fraction. Incomplete glycosylation was unlikely to be the cause of impaired

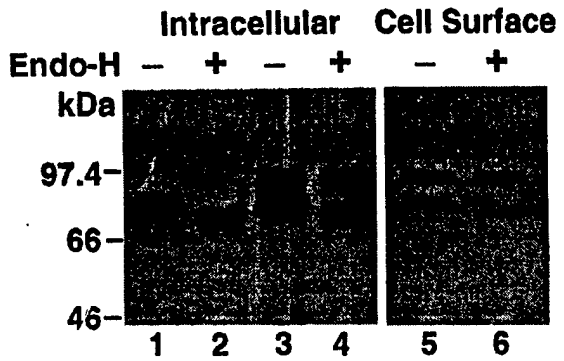

Fig. 6. Endo-H digestion of intracellular and cell surface associated MMP-9. Immunoprecipitates of the pulse-chase samples (*Lanes 1* and *2*, 0-min chase; *Lanes 3* and *4*, 30-min chase) with anti-MMP-9 antibodies or of surface-biotinylated (*Lanes 5* and *6*) TPA-treated MCF10A cells were digested with (*Lanes 2, 4,* and *6*) or without (*Lanes 1, 3,* and *5*) endo-H as described in "Materials and Methods." The samples were resolved by 7.5% SDS-PAGE analysis under reducing conditions followed by detection by autoradiography (intracellular) or streptavidin-HRP and the ECL detection system (cell surface).

secretion of the M_r 85,000 form because in the presence of tunicamycin, a glycosylation inhibitor, MCF10A cells secreted a lower molecular weight form of latent MMP-9. This suggests that the M_r 85,000 species of MMP-9 was retained on the PM by a unique interaction.[4] The pulse-chase studies also showed significant differences between the processing of MMP-9 and TIMP-1. Whereas the precursor form of TIMP-1 was rapidly processed and secreted into the medium, both the precursor MMP-9 form and a significant portion of the mature enzyme remained cell associated. Similar to MMP-9, TIMP-2 showed a codistribution between the cells and the medium, suggesting that the presence of TIMP-2 in the cellular fraction, after a 2-h chase period, was the result of secreted inhibitor that subsequently bound to the surface possibly via MT1-MMP (3, 10, 47). Indeed, MCF10A cells produce MT1-MMP and after synthesis this enzyme was intracellularly processed to a lower molecular weight form that remained in the cellular compartment. Endo-H digestion of the intracellular and cell surface-bound MMP-9 enzymes demonstrated that both the intracellular and the cell surface M_r 85,000 forms were endo-H sensitive, whereas the mature M_r 92,000 enzyme was endo-H resistant. Thus, differences in molecular weight between the cell surface MMP-9 species are consistent with differences in glycosylation and are not likely the result of a COOH-terminal truncation of the surface M_r 85,000 form. Taken together, the results of the pulse-chase experiments and endo-H digestions were consistent with the M_r 85,000 form on the cell surface being the precursor form of MMP-9. The functional significance of the cell surface association of the M_r 85,000 precursor form remains unknown. However, a previous study showed that unglycosylated MMP-9 remains capable of autocatalytic processing and catalytic activity (52). Thus, the cell surface-bound M_r 85,000 form would be expected to be functionally competent and, as such, to participate in cell surface proteolysis. The localization of the M_r 85,000 form of MMP-9 on the cell surface may represent the targeting of the endo-H sensitive MMP-9 precursor form to the PM, a process likely to be related to alterations in intracellular processing and trafficking in response to TPA. In contrast, the cell surface association of the mature MMP-9 may represent enzyme binding after secretion.

In vitro expression of MMP-9 has been shown to be induced by various cytokines (43) and growth factors (41, 42) and by cocultures of stroma cells with tumor cells (35, 44). Although most of the studies on MMP-9 induction examined the expression of the enzyme in the medium, a recent study with SKBR3 breast cancer cells cocultured with rat embryo fibroblasts showed that MMP-9 was not present in the detergent phase of Triton X-114 cell extracts (53). In agreement with these results, our studies showed that the M_r 92,000 MMP-9 form behaves like a peripheral membrane protein, because we were unable to detect it in the detergent phase. The hydrophilic nature of the MMP-9 association with the PM was also demonstrated after treatments of the PM with either 1 M NaCl, 0.1 M Na_2CO_3, or Triton X-114. Yet, a readily detectable fraction of both MMP-9 forms could not be released from the PM. This was most evident with the M_r 85,000 species suggesting a tighter, likely hydrophobic interaction with the PM, although the molecular mechanism involved remains to be elucidated.

A unique property of latent MMP-9 is its ability to form a noncovalent complex with TIMP-1 (1, 12, 25, 36, 37, 40). This interaction is fundamental for the regulation of MMP-9 activation and catalytic activity. Analysis of the cellular distribution of TIMP-1 after TPA treatment of MCF10A cells indicated that little or no inhibitor was present on the cell surface, unlike MMP-9. Indeed, TIMP-1 could not be detected in the immunoprecipitates of surface-biotinylated cells with anti-MMP-9 antibodies and was barely detected in the purified PM fractions, suggesting that neither of the surface-bound MMP-9 forms was forming a complex with TIMP-1. In contrast, immunoprecipitation with anti-MMP-9 antibodies and gelatin-agarose chromatography of the medium of the TPA-treated cells clearly demonstrated that TIMP-1 was in complex with MMP-9, as reported in previous studies (22, 25, 40). Moreover, immunoprecipitation of the medium with the anti-TIMP-1 antibodies, after removal of the MMP-9/TIMP-1 complex, showed the presence of free TIMP-1, suggesting that the inhibitor was in molar excess of MMP-9. The low amounts of TIMP-1 detected on the PM were unlikely to be the result of inhibitor dissociation during sample preparation because mild conditions were used during subcellular fractionation. In fact, disruption of the noncovalent binding of TIMP-1 to MMP-9 requires harsh conditions and can be accomplished, among other methods, with 0.1% trifluoroacetic acid (34), 20 mM HCl,[5] or 1% SDS. Also, a MMP-9/TIMP-1 recombinant complex could not be dissociated under the conditions used here to identify cell surface-associated MMP-9. The reason(s) for the lack of detection of a TIMP-1/MMP-9 complex on the cell surface in spite of the high level of TIMP-1 in the medium are presently unknown. It is possible that binding of MMP-9 to the cell surface may somehow interfere with TIMP-1 binding to the proenzyme form, suggesting that the association of MMP-9 with the cell surface may be mediated by the COOH-terminal domain. Alternatively, surface binding of MMP-9 may induce a conformational change, rendering its COOH-terminal domain inaccessible to the inhibitor. Further studies are required to distinguish between these possibilities. Nevertheless, the cell surface-association of MMP-9 free of TIMP-1 may have profound implications for activation and catalytic activity. Binding of TIMP-1 to the latent form of MMP-9 has been shown to alter activation by stromelysin-1 (37–39), plasmin (36, 37), and MMP-2 (45). Also, binding of TIMP-1 to activated MMP-9 inhibits enzymatic activity (37, 38, 45). Therefore, whereas in the extracellular space, the fate of latent MMP-9 would be determined by its interactions with TIMP-1, on the cell surface, the lack of available inhibitor would be expected to alter the activation and catalytic properties of MMP-9. It should be noted that in MCF10A cells, cell surface association of MMP-9 did not result in enzyme activation, possibly due to the lack of activators in these cells. Thus, the binding of MMP-9 to MCF10A cells is not likely to be mediated by a PM-bound activator as reported with MMP-2 (10,

[4] M. Toth, unpublished observation.

[5] M. Olson, unpublished observation.

55). The cell surface association of MMP-9, however, may render the enzyme accessible to the action of MMP-9 activators present in the tissue or in neighboring cells (36, 37, 45, 47, 50, 56, 57). Understanding the mechanisms that regulate the cell surface interactions of MMP-9 and elucidation of the molecule(s) responsible for binding would provide new insight on the regulation of ECM turnover in normal and malignant processes mediated by MMP-9.

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Carbohydrate-Mediated Regulation of Matrix Metalloproteinase-2 Activation in Normal Human Fibroblasts and Fibrosarcoma Cells

David C. Gervasi,* Avraham Raz,*† Marie Dehem,* Maozhou Yang,‡ Markku Kurkinen,*‡ and Rafael Fridman*[1]

*Department of Pathology, †Tumor Progression and Metastasis, Karmanos Cancer Institute, and ‡Center for Molecular Medicine and Genetics, Wayne State University, Detroit, Michigan 48201

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Matrix metalloproteinase-2 (MMP-2) is activated on the cell surface by membrane type 1-MMP (MT1-MMP). Activation of proMMP-2 is induced *in vitro* by concanavalin A (ConA). The regulation of proMMP-2 activation is, however, not yet fully understood. We investigated the effect of plant lectins, carbohydrates and inhibitors of the cytoskeleton on proMMP-2 activation in normal (HLF1) and malignant fibroblast (HT1080) cells. Native ConA induced proMMP-2 activation in both cell types while dimeric succinyl-ConA had no effect, suggesting that receptor clustering is involved in activation. Wheat germ agglutinin (WGA) also induced proMMP-2 activation. N-acetyl-D-glucosamine (GlcNac) inhibited the effects of ConA and WGA while mannose only inhibited ConA-induced proMMP-2 activation. Mannose also inhibited the expression of MT1-MMP mRNA induced by ConA. Cytochalasin B and colchicine had no effect on the ConA induction of proMMP-2 activation. These studies help to define some of the cellular and molecular mechanisms for the induction of proMMP-2 activation.

MMP-2 (gelatinase A, 72 kDa type IV collagenase) is a secreted endopeptidase able to hydrolyze several components of the extracellular matrix including basement membrane collagen IV and has been associated with tumor invasion and metastasis (1,2). MMP-2 also degrades non-collagenous proteins like galectin-3 (3), β-amyloid (4) and fibroblast growth factor receptor-1 (5). Thus, the broad specificity of MMP-2 may play a role in the regulation of various cellular activities. Like other members of the MMP family, MMP-2 is secreted in a latent form that is activated by a sequential cleavage of the N-terminal propeptide domain resulting in the generation of two active species of 62 and 59 kDa (6) and activation is cell surface-dependent (6-8). This process could be induced in cultured cells by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (8), ConA (7), transforming growth factor-β (8) and a collagen substrate (9). Analysis of the activating factor in the plasma membranes resulted in the identification of a novel type of MMP, the membrane type-MMP (MT-MMP) (10, 11). MT-MMPs are a subclass of MMPs that contain a unique transmembrane domain and a RXXR motif downstream of the N-terminal propeptide that is recognized by furin-like enzymes and may serve as a cleavage site for intracellular activation. Currently, four different MT-MMPs were isolated from normal and tumor tissues (10,12-14). However, the specific role of each type of MT-MMP in MMP-2 activation remains to be determined.

An important event in the understanding of the cellular mechanisms involved in proMMP-2 activation was the discovery by Overall and Sodek (15) of the ability of ConA to modulate the expression and activation of MMP-2 in human fibroblast cells. ConA is a widely use plant lectin composed of four identical subunits that specifically binds to cell

[1] All correspondence should be addressed to: Dr. Rafael Fridman, Department of Pathology, Wayne State University, 540 E. Canfield Avenue, Detroit, MI 48201. Fax: (313) 577-8180. E-mail: rfridman@med.wayne.edu.

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surface glycoproteins containing branched mannose residues. In mammalian cells, binding of ConA to cell surface glycoconjugates produces a variety of cellular responses including agglutination, cell proliferation, and clustering and internalization of cell surface receptors. In many aspects, the responses induced by ConA mimic the action of physiologically relevant ligands. ConA has been used as an important tool for defining some of the cellular mechanisms involved in the activation of MMP-2. For example, ConA-induced activation of proMMP-2 has been shown to require protein synthesis (16). In addition, ConA enhances the expression of MT1-MMP mRNA in breast (16) and cervical (17) cancer cells and MMP-2 mRNA in primary human fibroblasts (15). Like ConA, TPA induces MMP-2 activation in a variety of cultured cells (18). In the present study, we utilized lectins and TPA to further define some of the factors required for MMP-2 activation in HLF1 and HT1080 cells. Specifically, we investigated the carbohydrate specificities and involvement of the cytoskeleton in the activation of proMMP-2 induced by ConA and TPA and their effect on MT1-MMP mRNA and protein expression in both cell types.

MATERIALS AND METHODS

Cell culture. Human HT1080 fibrosarcoma (CCL-121) cells and normal human lung HLF1 fibroblasts (CCL-153) were obtained from American Type Culture Collection (Rockville, MD). The cell lines were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum and antibiotics and maintained in a 5% CO_2 incubator at 37°C.

Reagents and chemicals. ConA, WGA, succinyl-ConA, TPA, cytochalasin B, colchicine, D(+) mannose, N-acetyl-D-glucosamine (GlcNac), N-acetyl-D-galactosamine (GalcNac), and D-galactose were all purchased from Sigma Co (St. Louis, MO). The lectins were all dissolved in serum-free DMEM containing 25 mM Hepes. The TPA stock solution was made in ethanol to a final concentration of 2 mM. Cytochalasin B and colchicine stock solutions were prepared in dimethyl sulfoxide (DMSO) to final concentration of 2 and 2.5 mM, respectively.

Cell treatments. Cells were grown to 80% confluence in 24-well plates in complete media. At the time of treatment, the growth media was removed and the cells were washed twice with warm serum-free DMEM. Then, 0.3 ml/well of serum-free DMEM containing various concentrations of lectins or TPA (100 nM final concentration) were added to the cells for a 16 hr incubation period. In the case of TPA, the final concentration of ethanol added to the cells was 0.005% or 0.05 μl/ml. With cytochalasin B and colchicine the final concentration of DMSO was 0.95 and 0.08% (v/v), respectively. In each case, the same amount of vehicle was added to control cultures. The media was then collected, briefly centrifuged (14,000 rpm, 5 min) to remove cell debris and immediately analyzed by gelatin zymography. After each treatment, cell viability was determined by trypan blue exclusion.

Zymography. Gelatin-zymography was performed using 10% SDS-polyacrylamide gels containing 1% gelatin (18). Briefly, supernatants (15-20 μl) were mixed with 4X Laemmli sample buffer without reducing agents and without heating and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). At the end of the electrophoretic run, the gels were incubated (20 min., 22°C) in a solution of 2.5% Triton-X100 in H_2O and then washed in distilled H_2O, for another 20 min. The gels were then incubated (16 hrs, 37°C) in 50 mM Tris-HCl, 5 mM $CaCl_2$, pH 8 and then stained with 0.25% Coomassie Blue in a solution of 10% methanol and 5% acetic acid. Bands of gelatinolytic activity detected as cleared bands against the blue-stained gelatin background were visualized after destaining the gels with 10% methanol-5% acetic acid.

Northern blot analysis. Total RNA was extracted from untreated or treated HT1080 and HLF1 cells using the RNeasy Total RNA kit (Qiagen, Chatsworth, CA). Five μg RNA from each sample were fractionated on a 1% agarose gel in the presence of formaldehyde and then transferred onto a nylon membrane (Hybond-N, Amersham, Aylesbury, UK) followed by fixation to the membrane using an optimized UV crosslinking procedure. The blot was prehybridized at 42°C for 3 hr followed by hybridization at 42°C for 18 hr with a ^{32}P-labeled human cDNA probe for MT1-MMP (1.75 kb). Then, the blot was washed twice (15 min. each) with 0.1X SSC-0.1% SDS at 65°C and autoradiographed at −80°C. RNA loading was normalized using the signal obtained with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. Quantitation of signals was performed using an Ambis™ Radioanalytic Imaging System.

Immunoblot analysis. Analysis of MT1-MMP protein expression was performed in untreated and treated HT1080 and HLF1 cells extracted with the byphasic detergent Triton X-114. Briefly, cells were lysed with 1.5% Triton X-114 in Tris buffered saline (TBS, 50 mM Tris-HCl pH 7.5, 150 mM NaCl) containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonylfluoride (PMSF) and 5 mM EDTA. The cell extracts were then briefly (5 min) centrifuged (14,000 rpm, 4°C), and the supernatants were warmed (2 min., 37°C) and centrifuged (14,000 rpm, 22°C) to separate the detergent and aqueous phases. The detergent phase of each sample was diluted (4-fold) with distilled water

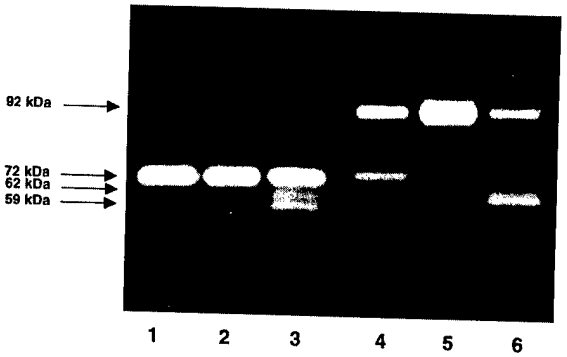

FIG. 1. Effect of Con and TPA on proMMP-2 activation in HT1080 and HLF1 cells. HLF1 (lanes 1-3) and HT1080 (lanes 4-6) cells were untreated (lanes 1 and 4) or treated (12 hrs) with either TPA (100 nM) (lanes 2 and 5) or ConA (10 μg/ml) (lanes 3 and 6) in serum free media. The media was then collected and analyzed by gelatin-zymography. The molecular weights in the left represent the apparent molecular mass of latent and active MMP-9 (92 kDa) and MMP-2 (62 and 59 kDa) forms.

followed by the addition of sample buffer. The samples were then subjected to SDS-PAGE and transferred to BA-S 85 nitrocellulose membrane (Schleicher & Schuell, Keene, NH). Blots were blocked with 3% bovine serum albumin and 3% nonfat dry milk in 100 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.02% NaN_3 (blotto). The blots were then incubated with a polyclonal rabbit antibody raised against a synthetic peptide (RFNEELRAVDSEYPNIK) derived from the amino acid sequence of human MT1-MMP (10) and produced by Genetics Research, Inc. (Huntsville, AL) based on antigenic site predictions of Dr. Steve Ledbetter (Genzyme, Cambridge, MA). The blots were incubated with anti-MT1-MMP antibody diluted (1:5000) in 20 mM Tris-HCl, pH 7.5, containing 137 mM NaCl and 0.1% Tween-20 and then incubated with a goat anti-rabbit polyclonal antibody. The immunodetection of the antigen was performed using the ECL kit (Amersham) according to the manufacturer's instructions.

RESULTS

Effect of ConA and TPA on proMMP-2 activation. We initially confirmed the ability of ConA and TPA to induce activation of proMMP-2 in HT1080 and HLF1 cells. As shown in Figure 1, ConA induces proMMP-2 activation in HLF1 cells resulting in the generation of the 62- and 59-kDa forms (Figure 1, lane 3) confirming previous reports (6,18) while TPA had no effect (Figure 1, lane 2). TPA treatment of HT1080 cells, which constitutively produce both proMMP-9 and proMMP-2 (Figure 1, lane 4), resulted in the secretion of higher amounts of proMMP-9 (19) and the appearance of the active 62-kDa form of MMP-2 (Figure 1, lane 5). Dose dependent experiments demonstrated a maximal activation of proMMP-2 at ConA concentrations of 5-10 μg/ml for both HLF1 (Figure 2, lanes 5 and 6) and HT1080 cells (Figure 3, lane 3). Higher doses of ConA (20-30 μg/ml) did not alter the pattern of proMMP-2 activation. It should be noted that with any given dose of ConA, conversion of the latent form to the active species was never complete after a 24 hr incubation period.

Effect of Succinyl-ConA. Succinyl-ConA is a dimeric protein lectin that maintains the carbohydrate binding specificity of native ConA but does not induce ligand mobility or clustering on the cell surface. We compared the effects of ConA and succinyl-ConA on proMMP-2 activation in HLF1 and HT1080 cells. As shown in Figures 2 and 3, treatment of cells with succinyl-ConA for 24 hrs consistently failed to induce a detectable proMMP-2 activation in both cell types even at doses as high as'25 μg/ml. This suggests that clustering of cell surface mannose-containing glycoproteins are required for the ConA-induced activation of proMMP-2 in both cell types.

Effect of WGA and sugars on proMMP-2 activation. We wished to define some of the cell surface carbohydrate moieties involved in the signalling process leading to proMMP-2 activa-

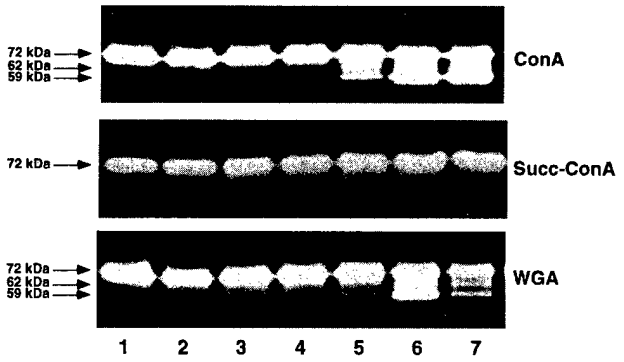

FIG. 2. Effect of ConA, Succinyl-ConA and WGA on proMMP-2 activation in HLF1 cells. HLF1 cells were treated (12 hrs) with increasing concentrations (lane 1: O; lane 2: 0.62; lane 3: 1.25; lane 4: 2.5; lane 5: 5; lane 6: 10 and lane 7: 20 μg/ml) of either ConA, succinyl-ConA or WGA in serum free media. The media was then collected and subjected to gelatin-zymography as described in Materials and Methods. The molecular weights represent the apparent molecular mass of latent and active MMP-2 forms.

tion by ConA. To this end, we compared the effects of ConA and WGA which differ in their carbohydrate recognition specificities. ConA binds α-mannose, glucose and α-GlcNac and WGA binds β-GlcNac and sialic acid. In addition, we tested the ability of purified sugars to block the effect of these lectins on proMMP-2 activation. As shown in Figures 2 and 3, exposure of HLF1 (Figure 2) and HT1080 cells (Figure 3) to WGA (5-20 μg/ml) resulted in the activation of proMMP-2 in pattern similar to that obtained with ConA. Addition of mannose in the presence of 10 μg/ml ConA to HT1080 cells lead to a significant reduction in the amount of the 62-kDa form and inhibition of formation of the 59-kDa species (Figure 4, lanes 3-6, upper panel). At 400 nM, mannose caused a non-specific reduction in the secretion of both MMPs. Addition of GlcNac in the presence of ConA (Figure 4, lanes 7-9, upper panel) also inhibited proMMP-2 activation. Mannose had no apparent effect on the WGA-induced

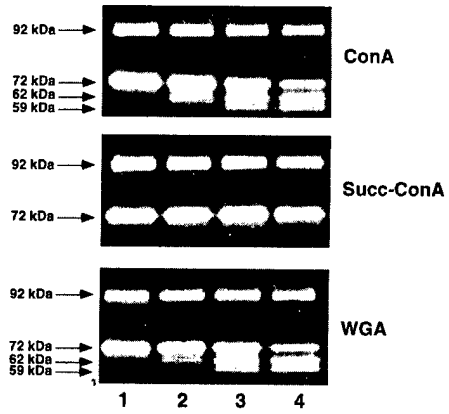

FIG. 3. Effect of ConA, Succinyl-ConA and WGA on proMMP-2 activation in HT1080 Cells. HT1080 cells were treated (12 hr) with, lane 1:0; lane 2:5; lane 3: 10 and lane 4: 20 μg/ml of either ConA, succinyl-ConA or WGA in serum free media. The media was then collected and subjected to gelatin-zymography as described in Materials and Methods. The molecular weights represent the apparent molecular mass of latent and active MMP-9 and MMP-2 forms.

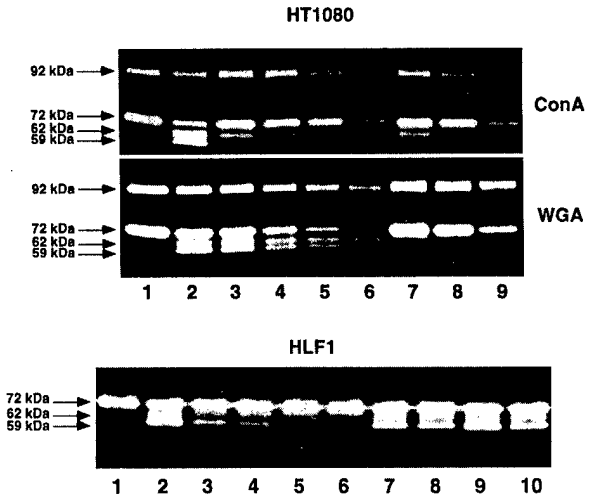

FIG. 4. Effect of mannose, GlcNac, GalcNac and galactose on proMMP-2 activation induced by ConA and WGA. HT1080 cells were treated with serum free media containing ConA (10 μg/ml) or WGA (10 μg/ml) (lanes 2-9) in the absence (lane 2) or presence of either mannose (lanes 3: 50 mM; lane 4: 100 mM; lane 5: 200 mM; lane 6: 400 mM) or GlcNac (lane 7: 50 mM; lane 8: 100 mM; lane 9: 200 mM). HLF1 cells were treated with ConA (10 μg/ml) (lanes 2-5 and 7-10) in the presence of either mannose (lane 3: 100 mM; lane 4: 200 mM; lane 5: 400 mM), GalcNac (lane 7: 200 mM; lane 8: 400 mM) or galactose (lane 9: 200 mM; lane 10: 400 mM). In lane 6, HLF1 cells received only 100 mM mannose. The media was then collected and subjected to gelatin-zymography as described in Materials and Methods.

proMMP-2 activation (Figure 4, lanes 3-6, lower panel). In contrast, GlcNac strongly inhibited the induction of proMMP-2 activation by WGA (Figure 4, lanes 7-9, lower panel). Similar inhibitory results were observed in HLF1 cells incubated with WGA (not shown) or ConA in the presence of mannose (Figure 4, lanes 3-5) or GlcNac (not shown). Treatment of HT1080 (not shown) or HLF1 (Figure 4) cells with ConA in the presence of GalcNac (Figure 4, lanes 7 and 8) or galactose (Figure 4, lanes 9 and 10) had no effect on activation of proMMP-2.

Effects of plant lectins and TPA on MT1-MMP expression. The activation of proMMP-2 induced by ConA and TPA has been previously shown to be mediated by MT1-MMP (10,11). Therefore, we examined the mRNA and protein expression of MT1-MMP in HLF1 and HT1080 cells treated with lectins (Con A or WGA) or TPA. As shown in Figure 5, both untreated HT1080 (lane 1) and HLF1 (lane 5) cells constitutively produce the 4.5 kb MT1-MMP mRNA. Exposure of HT1080 cells to TPA (Figure 5, lane 2) induced a 2-fold induction in MT1-MMP mRNA expression in agreement with a previous study (20). ConA induced a 1.5 fold expression over the basal levels and WGA had no significant effect (Figure 5, lanes 3 and 4, respectively). In HLF1 cells, Con A (Figure 5, lane 7) and WGA (Figure 5, lane 8) treatment resulted in a 3- and a 2.6 fold induction of MT1-MMP mRNA expression over basal level, respectively. The induction of MT1-MMP mRNA by ConA in HLF1 cells was abolished by 100 mM mannose (Figure 5, lane 9) consistent with the inhibitory effect of mannose on ConA-induced proMMP-2 activation (Figure 4). Interestingly, TPA treatment of HLF1 cells [which failed to induce proMMP-2 activation (Figure 1)], resulted in a 1.9 fold induction of MT1-MMP mRNA expression (Figure 5, lane 6). The expression of the MT1-MMP polypeptide was also examined by immunoblot analysis using a polyclonal antibody against a synthetic peptide derived from the human MT1-MMP sequence. This antibody reacts specifically with a protein of 63-kDa in purified plasma membranes of TPA-treated HT1080 cells (Figure 6, lane 1). Since MT1-

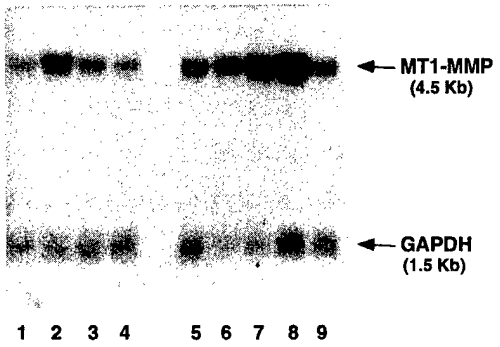

FIG. 5. Expression of MT1-MMP mRNA in HT1080 and HLF1 Cells. Cultures of HT1080 (lanes 1-4) and HLF1 (lanes 5-9) cells were treated with either 100 nM TPA (lanes 2 and 6), 10 μg/ml ConA (lanes 3 and 7), 10 μg/ml WGA (lanes 4 and 8) or 10 μg/ml ConA in the presence of 100 mM mannose (lane 9) in serum free media. Parallel cultures were established without treatment (lanes 1 and 6). After 16 hrs, total RNA was extracted, electrophoresed on a 1% agarose gel, blotted to nitrocellulose paper and probed with cDNA probes to MT1-MMP and GAPDH.

MMP is a plasma membrane-bound enzyme, we examined the expression of MT1-MMP in the detergent phase of Triton X-114 solubilized cells as described in Materials and Methods. This detergent extraction procedure resulted in a better resolution of the MT1-MMP protein. As shown in Figure 6, lane 2, extracts of untreated HLF1 cells did not show detectable levels of MT1-MMP protein, however, when the cells were treated with either ConA (Figure 6, lane 3) or WGA (Figure 6, lane 4), expression of MT1-MMP was readily detected. In HT1080 cells, expression of MT1-MMP was observed in untreated cells (Figure 6, lane 5) and its expression was apparently unaltered by treatment with ConA and WGA (Figure 6, lanes 6 and 7, respectively).

Effect of cytochalasin B and colchicine on ConA-induced activation of proMMP-2. We wished to test whether the integrity of the cytoskeleton plays a role on proMMP-2 activation after treatment with ConA. To this end, we examined the effects of cytochalasin B, which disrupt microfilament function and architecture, and colchicine, which disaggregates the microtubules. HLF1 and HT1080 cells (Figure 7, only HT1080 cells are shown) were treated (30

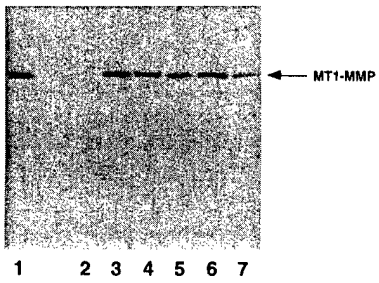

FIG. 6. Immunoblot analysis of MT1-MMP. HLF1 (lanes 2-4) and HT1080 (lanes 5-7) cells (2×10^5 cell/well) were either untreated (lanes 2 and 5) or treated with 10 μg/ml ConA (lanes 3 and 6) or 10 μg/ml WGA (lanes 4 and 7) for 16 hrs in serum free media. Cells were then lysed with 1.5% Triton X-114 in Tris buffered saline to obtain the detergent and aqueous phase as described in the Materials and Methods. The detergent phase of each sample was then subjected to SDS-PAGE in 12% polyacrylamide gels under reducing conditions followed by transfer to a nitrocellulose membrane. MT1-MMP (63 kDa) was detected using a polyclonal antibody and the ECL kit. Lane 1 shows the expression of MT1-MMP in purified plasma membranes of TPA-treated HT1080 cells.

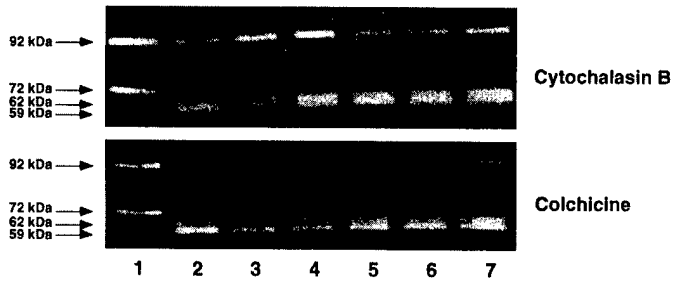

FIG. 7. Effect of cytochalasin B and colchicine in proMMP-2 activation. HT1080 cells were untreated (lane 1) or treated (30 min, 37°C) with various concentrations of either cytochalasin B (lane 3: 2.5 μM; lane 4: 5 μM; lane 5: 10 μM; lane 6: 20 μM) or colchicine (lane 3: 0.25 μM; lane 4: 0.5 μM; lane 5: 1 μM; lane 6: 2 μM) before addition of 10 μg/ml of ConA (lanes 2-7). Lane 7 shows cells treated only with ConA and 0.08% (colchicine) or 0.95% (cytochalasin B) DMSO (final concentration), the vehicle of the two drugs.

min, 37°C) with various concentrations of either cytochalasin B or colchicine before addition of 10 μg/ml ConA for another 16 hrs. The media was collected and then analyzed by zymography. Neither cytochalasin B nor colchicine had an effect on the ConA-induced activation of proMMP-2 in HT1080 (Figure 7) and HLF1 cells (not shown) suggesting that the MT1-MMP and its putative activator(s) are not associated with the cytoskeleton.

DISCUSSION

In this study we investigated some of the cellular processes thought to play a role in the activation of proMMP-2 by ConA and TPA in two established normal (HLF1) and malignant (HT1080) fibroblast cell lines. As previously reported with other cells (7,8,16), ConA is an effective inducer of proMMP-2 activation in both cell lines generating the 62- and 59-kDa active forms. In contrast to ConA, TPA-mediated activation is cell type specific since only HT1080 cells responded. A lack of activation after TPA treatment in normal cells was also reported in primary human gingival fibroblasts (15) and in WI-38 fibroblasts (8) both of which respond to ConA. However, other study demonstrated that embryonic lung fibroblasts (CCL-137) do not respond to ConA (21). The activation of proMMP-2 induced by TPA and ConA has been shown to be mediated by MT1-MMP, a recently discovered MMP that is bound to the plasma membrane (10,11). In this study, we examined the effects of ConA and TPA on MT1-MMP expression and found a complex relationship between MT1-MMP expression and proMMP-2 activation. Although TPA caused a 2-fold increase in MT1-MMP mRNA levels in both cell types, only in HT1080 cells proMMP-2 was activated after TPA treatment, suggesting that in HLF1 cells induction of MT1-MMP expression alone is not sufficient to induce proMMP-2 activation. This was not the case after ConA treatment where we found a positive correlation between induction of MT1-MMP expression and proMMP-2 activation in both cell types as previously shown with a breast cancer cell line (16). Consistently, addition of mannose, the competitive sugar, inhibited ConA-mediated proMMP-2 activation and MT1-MMP mRNA expression. Thus, in HLF1 and HT1080 cells, transcriptional regulation of MT1-MMP expression is a necessary step in the cascade of events leading to proMMP-2 activation. However, in spite of the expression of MT1-MMP mRNA, untreated HLF1 cells did not show presence of detectable amounts of MT1-MMP protein and consequently did not activate proMMP-2. In untreated HT1080 cells, expression of MT1-MMP protein was not sufficient to cause proMMP-2 activation suggesting that the activation of proMMP-2 requires both active

transcription of MT-MMP-1 mRNA expression and another yet unidentified element(s) that, depending on the cell type and signal, may include synthesis and processing of MT1-MMP protein into an active membrane-bound enzyme or production of a co-factor as suggested by Yu et al (16) for breast cancer cells. A recent study reported that induction of MT1-MMP mRNA expression in an embryonic lung fibroblast cell line by TPA or ConA did not result in activation of proMMP-2 consistent with the need of additional co-operating factor(s) for this process (21). It is possible, therefore, that the differential response to ConA, in terms of proMMP-2 activation, between fibroblast cell lines (21), in spite of the similar induction of MT1-MMP expression, may be related to the presence or absence of this additional co-factor(s).

To define the signaling pathway leading to proMMP-2 activation after exposure to lectins we compared ConA with succinylated ConA and found that proMMP-2 activation did not take place when either cell type was treated with succinylated ConA even at high concentrations. Since these two variants posses the same carbohydrate specificity, differences in response may be related to the dimeric conformation of succinyl ConA as compared to the tetramer configuration of ConA. The latter facilitates the clustering of glycosylated ligands on the cell surface that may initiate activation of intracellular signalling pathways. The carbohydrate requirement for the induction of proMMP-2 activation was examined using ConA and WGA. We have found that both lectins were equally active in HLF1 and HT1080 cells suggesting that GlcNac, mannose and sialic acid oligosaccharide-containing glycoprotein(s) may all be involved in this process. Consistently, the specific competitive sugars, i.e., mannose and GlcNac but not galactose or GalcNac inhibited proMMP-2 activation demonstrating that the effect of ConA is mediated by carbohydrate recognition. However, the WGA effect was inhibited only by the presence GlcNac suggesting that the WGA-induced activation of proMMP-2 is mediated by its binding to a GlcNac-containing cell surface molecule. The relevance of the carbohydrate moieties was further demonstrated by the ability of mannose to specifically inhibit the induction of MT1-MMP mRNA expression by ConA. In agreement with this result, a previous study showed that induction of MMP-2 mRNA by ConA in human gingival fibroblasts was inhibited by α-methyl-D-mannopyranoside (15) suggesting that activation is not limited to the plasma membrane compartment but involves a signal transduction pathway from the cell surface to the nucleus.

While ConA clearly induces proMMP-2 activation in fibroblast cells, it also causes significant changes in cell morphology with cells adopting a rounded shape compared to the classical elongated spindle shape of untreated fibroblasts (15). Thus, ConA treatment may affect the organization of the cytoskeleton. Another study showed that proMMP-2 activation in human breast cancer cell lines could be induced by culturing the cells on a 3-dimensional gel of collagen I (9). This substrate is well known for its ability to influence many cellular activities involving changes in cell shape including adhesion, spreading, contraction, and cell motility. Since ConA and TPA treatment may induce translocation of MT1-MMP protein to the plasma membrane, it was noteworthy to find that both cytochalasin B and colchicine, two agents known to disrupt microfilaments and microtubules, respectively, had no effect on proMMP-2 activation in HLF1 and HT1080 cells. This lack of effect is also intriguing giving the fact that succinyl-ConA, which does not induce receptor clustering, was inactive in promoting proMMP-2 activation. These results suggest that translocation and localization of MT1-MMP to the plasma membrane may not be dependent on the organization of the cytoskeleton. Likewise, the intracellular signalling pathway activated by ConA and TPA remains functional under conditions that disrupt the organization of the cytoskeleton. In summary, the results presented here show that normal and tumorigenic human fibroblasts respond differently to inducers of proMMP-2 activation

and that activation involves a signal transduction pathway of carbohydrate recognition and clustering ligand(s) and the action of a yet uncharacterized factor.

ACKNOWLEDGMENTS

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EPIDERMAL GROWTH FACTOR AND AMPHIREGULIN UP-REGULATE MATRIX METALLOPROTEINASE-9 (MMP-9) IN HUMAN BREAST CANCER CELLS

Sudhir B. KONDAPAKA, Rafael FRIDMAN and Kaladhar B. REDDY*
Department of Pathology, Wayne State University, Detroit, MI

The EGF family of proteins encompasses several polypeptides such as epidermal growth factor (EGF), transforming growth factor alpha (TGFα), amphiregulin (AR) and heregulin (HRG-β1). These polypeptides regulate proliferation in breast cancer cells through interaction with membrane receptors. It has been previously shown that high EGF receptor number correlates with aggressive behavior and increased metastasis in human breast cancer. In the present study, we investigated the association between EGF and EGF-like ligand-induced DNA synthesis and secretion of MMP-9 and MMP-2 in metastatic SKBR-3 and non-metastatic MCF-7 breast cancer cells. Exposure of SKBR-3 cells to EGF or AR induces expression of MMP-9 but has no effect on MMP-2 secretion. In contrast to EGF and AR, HRG had no effect on gelatinase induction. None of the EGF polypeptides had any effect on gelatinase induction in MCF-7 non-metastatic breast cancer cells. While a relatively specific inhibitor of EGF receptor tyrosine kinase, PD 153035, inhibited EGF-, AR- and HRG-induced cell proliferation, it had no effect on MMP-9 induced by EGF and AR. Experimental evidence suggests that signaling mechanisms for cell proliferation and MMP-9 induction are mediated by different pathways down-stream of EGF receptor autophosphorylation or that low levels of EGF-induced signal that escape inhibition are sufficient to induce MMP-9 but unable to support cell proliferation. In addition, our results suggest that EGF and AR may modulate invasion of metastatic breast cancer cells by increasing the expression of MMPs. *Int. J. Cancer* 70:722–726, 1997.
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The EGFR and erbB2/HER2 receptor are members of the type I growth factor family of receptors whose over-expression has been shown to correlate with decreased disease-free survival and increased metastasis in advanced breast cancer (Sainsbury *et al.,* 1987; Khazaie *et al.,* 1993). Tumor cells generally exhibit a decreased requirement for growth factors when compared with their normal counterparts due to increased endogenous synthesis and secretion of polypeptide growth factors produced by the tumor itself and/or by the surrounding cells by an autocrine or a paracrine mechanism (Osborne and Arteaga, 1990). The over-expression of growth factors or their receptors would give growth advantage to the tumors expressing them, and indeed, this has been observed in many primary breast cancers and cell lines (Normanno *et al.,* 1994; Rajkumar and Gullick, 1994; Kokai *et al.,* 1989). These growth factors bind to specific plasma membrane receptors, activating the receptor tyrosine kinase activity that is essential for signal transduction and biological function (Reddy *et al.,* 1992; Heldin *et al.,* 1987; Kokai *et al.,* 1988). Furthermore, the over-expression of some of these mitogens can initiate *in vitro* transformation of both murine and human mammary epithelial cells (Ciardiello *et al.,* 1990, 1991).

Expression of MMPs, a family of zinc-dependant endopeptidases, has been associated with tumor cell invasion and metastasis due to the ability of these proteinases to hydrolyze a variety of extracellular matrix proteins. Two members of the MMP family, the 72-kDa (MMP-2, gelatinase A) and 92-kDa (MMP-9, gelatinase B) proteinases, have been shown to be highly expressed in breast tumors in both the stroma and the cancer cells (Monteagudo *et al.,* 1990; Davies *et al.,* 1993; Sato and Seiki, 1993). MMP-2 and MMP-9 are thought to play a role in the degradation of basement membrane collagen IV and, hence, may contribute to the invasive ability of breast cancer cells (Bae *et al.,* 1993). *In situ* hybridization studies have shown that the expression of MMP-2 mRNA is mostly localized in the tumor fibroblasts, while MMP-9 mRNA was found to be expressed by epithelial cells and macrophages (Davies *et al.,* 1993; Polette *et al.,* 1993; Wilhelm *et al.,* 1989; Poulsom *et al.,* 1992; Fridman *et al.,* 1995; Okada *et al.,* 1995). Thus, several cellular constituents of the tumor may interact to carry out degradation of the extracellular matrix by modulating MMP expression. The mechanisms regulating gelatinase expression in breast tumors are unknown—in particular, those responsible for the expression of MMP-9 in breast cancer cells. Previous studies demonstrated that MMP-9 expression can be induced by a variety of stimuli, including phorbol ester (Wilhelm *et al.,* 1989), transforming growth factor-β, (Overall *et al.,* 1989) and interleukin-1β (Conca *et al.,* 1989), in different types of tumor cells. Previously, it was also shown that EGF can increase the motility and invasiveness of keratinocytes, suggesting a role of EGF in partially supporting the tumor metastatic processes (Lisa and Laurie, 1996). The role of other EGF-like ligands in MMP-2 and MMP-9 expression in human breast cancer is not known. In the present study, we examined the effects of EGF-like ligands on MMP-9 and MMP-2 expression in metastatic SKBR-3 and non-metastatic MCF-7 breast cancer cells. Here, we show that exposure of breast cancer cells to EGF and AR, which bind to EGFR, resulted in EGFR autophosphorylation, increased cell proliferation and induction of MMP-9. In contrast to EGF and AR, HRG, which binds to erbB3 and erbB4 receptors, had no effect on MMP-9 expression. Also, PD 153035, a tyrosine kinase inhibitor which inhibits cell proliferation, failed to inhibit MMP-9 induction by EGF. Our results suggest that EGF and AR may contribute to the invasion of breast cancer cells by modulating expression of MMP-9.

MATERIAL AND METHODS

Cells and cell culture

The SKBR-3 and MCF-7 cell lines were obtained from the ATCC (Rockville, MD). All breast cancer cells were cultured in DMEM (GIBCO, Gaithersburg, MD), supplemented with 5–10% FCS (GIBCO) and 10 nM insulin (GIBCO), as described previously (Reddy *et al.,* 1994). All cell lines were routinely tested for Mycoplasma contamination and found to be negative.

Growth factors, antibodies and tyrosine kinase inhibitor

EGF and TGFα were purchased from Collaborative Research (Lexington, MA). AR was purchased from R&D Systems (Minneapolis, MN), and HRG was provided by Dr. Hung (M.D. Anderson, Houston, TX). The tyrosine kinase inhibitor PD 153035 was provided by Dr. Fry (Parke-Davis, Ann Arbor, MI). Tyrosine kinase inhibitor stock solutions were made in DMSO and diluted to appropriate concentrations in culture medium prior to addition to the cells. An equivalent dilution of DMSO (0.1%) without the inhibitor served as a control. Antibodies to EGFR and anti-

Abbreviations: TGFα, transforming growth factor alpha; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; AR, amphiregulin; HRG, heregulin; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase.

*Correspondence to: Department of Pathology, Wayne State University, 540 E. Canfield Avenue, Detroit, MI 48201, USA. Fax: (313) 577-0057.

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phosphotyrosine were purchased from UBI (Lake Placid, NY). MMP-9 and TIMP-1 polyclonal antibodies were provided by Dr. Fridman (Wayne State University, Detroit, MI).

Western blot analysis

SKBR-3 cells were grown to near-confluence in 15-cm dishes in regular growth medium. The seeding medium was removed, cells were washed twice with PBS and the medium was replaced with phenol red-free and serum-free DMEM. Twenty-four hours later, the cells were pre-exposed to 40 nM PD-153035 for 2 hr and then treated with EGF or AR for 5 min. Cells were then washed with ice-cold PBS and scraped into lysis buffer (50 mM Tris-HCl [pH 7.6], 100 mM NaCl, 2 mM EDTA, 1% NP-40, 1 mM orthovanadate, 0.5% sodium deoxycholate, 10 mg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin). After removal of cell debris by centrifugation (14,000 *g*, 30 min), the protein concentration was estimated. Equal amounts of protein from different groups were subjected to EGFR immunoprecipitation or immediately boiled for 5 min in sample buffer. Immunoprecipitates were washed 3 times with HNTG (20 mM Tris-HCl [pH 7.6], 10% glycerol, 0.1% NP-40, 150 mM NaCl) and heated in loading buffer for 10 min (100°C). Proteins were resolved in 8% SDS-PAGE and transferred onto nylon membrane (MSI, Westboro, MA), then probed with the appropriate antibody (Reddy *et al.*, 1992). Blots were washed 3 times with TTBS (20 mM Tris-HCl [pH 7.6], 0.05% Tween 20, 100 mM NaCl) and incubated with a secondary antibody coupled to horseradish peroxidase. After 3 washes, the blot was developed using the ECL chemiluminescence system (Amersham, Arlington Heights, IL). The level of phosphorylated EGFR was determined by densitometric scanning of M_r 170,000 band.

DNA synthesis

Cells were plated in 24-well tissue culture dishes (Corning, Corning, NY) at a density of 1×10^5 cells per well in DMEM with 10% FCS. After 24 hr, the seeding media were removed, the cells washed twice with PBS and the cells incubated in 0.1% BSA and phenol red-free DMEM (1 ml/well). Twenty-four hours later, growth factors and/or tyrosine kinase inhibitor were added. After 18 hr, [^{3}H]thymidine (82.3 Ci/mmol; NEN, Boston, MA; 0.25 μCi in a volume of 25 μl) was added to each well for a 1 hr pulse. Cells were harvested, and the rate of DNA synthesis was estimated in triplicate by measuring the acid-precipitable radioactivity as described previously (Reddy *et al.*, 1994). Radioactivity was quantified in a Beckman (San Ramon, CA) LS 6000 liquid scintillation counter with an efficiency of 64%.

Gelatin zymography

Fifteen micrograms of protein from different groups were separated in 10% SDS-polyacrylamide gels co-polymerized with 0.1% gelatin. After electrophoresis, gels were rinsed twice in 2.5% Triton X-100 and incubated at 37°C for 20 hr in 1.5 M NaCl, 10 mM $CaCl_2$ and 50 mM Tris-HCl buffer (pH 7.5). Gels were stained with 0.5% Coomassie blue R250 and destained in 50% methanol and 10% acetic acid in H_2O. Gelatinolytic enzymes were detected as transparent bands on the blue background of Coomassie blue-stained gel.

RESULTS

EGF and AR induce MMP-9 in SKBR-3 breast cancer cells

We examined the effect of EGF, AR and HRG on the expression of MMP-2 or MMP-9 in SKBR-3 and MCF-7 cells. As shown in the zymogram of Figure 1*a*, untreated SKBR-3 cells do not secrete detectable levels of either MMP-2 or MMP-9. Exposure of these cells to as little as 5 ng/ml of EGF, however, resulted in secretion of the latent form of MMP-9 into the medium. Increasing EGF concentrations (50 and 500 ng/ml) did not change the level of MMP-9 secretion (Fig. 1*a*). Treatment of SKBR-3 cells with 1 ng/ml of AR also induced secretion of MMP-9 (data not shown). In contrast, HRG had no effect on MMP induction even after 72 hr (Fig. 1*b*). Neither of these mitogens induced MMP-2 expression in SKBR-3 cells or gelatinase expression in MCF-7 cells.

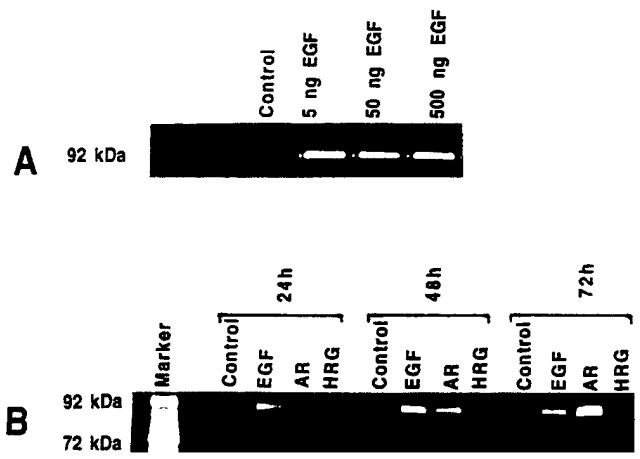

FIGURE 1 – EGF and AR up-regulate progelatinase B (MMP-9) in SKBR-3 breast cancer cells. SKBR-3 cells were grown in DMEM, with serum, to near confluence. The seeding medium was removed, cells were washed twice with PBS and medium was replaced with phenol red-free and serum-free DMEM. Twenty-four hours later, 50 ng/ml of EGF, AR and HRG were added to the culture. After an additional 24 hr, conditioned medium was collected and the presence of MMPs was evaluated by gelatin zymogram, as described in "Material and Methods". *(a)* Low concentrations of EGF (5 ng/ml) induce MMP-9. *(b)* Time course effects of EGF, AR and HRG. HRG had no effect on MMP-9 induction up to 72 hr.

Effect of EGF, AR and HRG on autophosphorylation of EGFRs and cell proliferation

To assess the functionality of EGF, AR and HRG on SKBR-3 cells, we examined the ability of these ligands to increase EGFR and erbB2 receptor autophosphorylation and cell proliferation. As shown in Figure 2*a*, both EGF and AR increased EGFR autophosphorylation in SKBR-3 cells when compared with untreated cells. In contrast, HRG had no effect. EGF and AR also caused phosphorylation of the erbB2 receptor, suggesting an EGF:erbB2 receptor heterodimerization. HRG, which predominantly binds to erbB3 and erbB4, marginally increased c-erbB2 receptor phosphorylation (Fig. 2*a*). In contrast to the differential effect of these ligands on type I receptor phosphorylation, they all promoted the proliferation of SKBR-3 cells (Fig. 2*b*).

Inhibition of EGFR autophosphorylation by a specific tyrosine kinase inhibitor

Previous studies demonstrated that PD 153035 is a relatively specific inhibitor of EGFR tyrosine kinase activity in a variety of target cells *in vitro* (Reddy *et al.*, 1992). We examined the effect of PD 153035 on EGFR phosphorylation in SKBR-3 cells. To this end, SKBR-3 cells were pre-incubated for 2 hr with PD 153035 prior to EGF (50 ng/ml) stimulation for 5 min and lysed. Western blot analysis using anti-phosphotyrosine antibodies demonstrated a dose-dependent inhibition of EGF-stimulated autophosphorylation of an M_r 170,000 band, representing EGFR (Fig. 3); half-maximal inhibition was observed at a concentration of about 40 nM of PD 153035.

Effect of PD 153035 on EGF- and AR-induced ^{3}H-thymidine incorporation and MMP-9 secretion

Since PD 153035 inhibited EGF-stimulated autophosphorylation, we examined its effect on EGF- and AR-stimulated ^{3}H-thymidine incorporation and on the ability of EGF to induce MMP-9 secretion. SKBR-3 cells were treated with EGF or AR in the presence or absence of 40 nM PD 153035 for 18 hr. Then, both the incorporation of ^{3}H-thymidine into the cells and the presence of MMP-9 in the media were examined. As shown in Figure 4*a*, both EGF and AR induced a 2- to 3-fold increase in ^{3}H-thymidine incorporation, which was blocked by PD 153035. Similar data

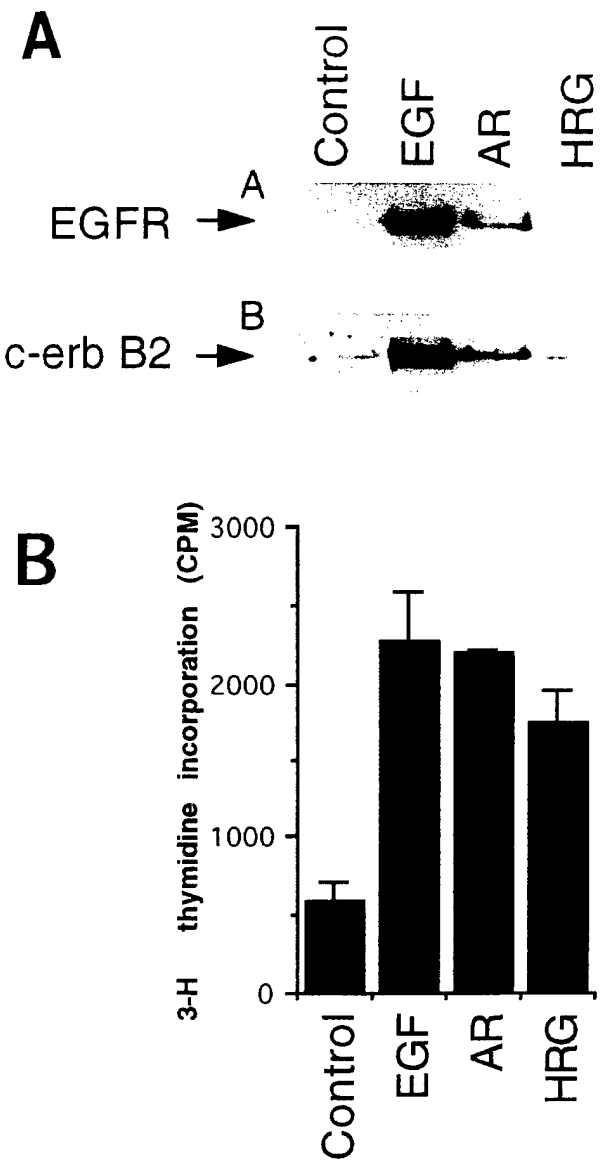

FIGURE 2 – Effect of EGF, AR and HRG on autophosphorylation of EGFRs and ^{3}H-thymidine incorporation. Cells were grown as shown in Figure 1; stimulated for 5 min with EGF, AR or HRG; and lysed. Western blot analyses were done by immunoprecipitating the protein lysate by anti-EGFR or anti-erbB receptor monoclonal antibodies, separated on SDS-PAGE and transferred to a nylon membrane, then probed with anti-phosphotyrosine antibody *(a) (b)* [^{3}H]thymidine incorporation by cells incubated for 18 hr with the ligands. Bars, mean ± SE of triplicate determinations. EGF, AR and HRG show the expected pattern of receptor autophosphorylation and cell proliferation.

were obtained with MCF-7 cells (Fig. 5). Cells incubated in the presence of PD 153035 (40 nM) for 24 hr remain viable, as indicated by their morphological appearance, their lack of floating cells and their ability to exclude Trypan blue (>90% viable). Interestingly, secretion of MMP-9 into the media of SKBR-3 cells induced by treatment with either EGF or AR was not affected by the presence of PD 153035, as determined by both zymography and Western blot analysis with a specific MMP-9 antibody (Fig. 4*b,c*).

DISCUSSION

The majority of breast carcinomas when compared with normal breast epithelium show over-expression of ligands of the EGF family, including TGFα, AR and cripto-1 (CR-1) (Normanno *et al.*, 1994). Furthermore, the introduction of rat or human TGFα cDNA into the germ line of transgenic mice promotes the appearance of hormone-dependent mammary adenocarcinomas (Matsui *et al.*, 1990; Jhappan *et al.*, 1990). These data support the hypothesis that growth factors are involved not only in cell proliferation but also in the pathogenesis of breast cancer. Accordingly, previous studies have shown that high levels of EGFR expression correlate with aggressive behavior and increased metastasis in human breast cancer (Sainsbury *et al.*, 1987; Khazaie *et al.*, 1993). Thus, expression of EGF-like ligands and their receptors in breast tumors may play a role in the expression of the metastatic phenotype. Our study was aimed at defining the role of EGF and EGF-like ligands and their receptors in the regulation of MMP-2 and MMP-9 in breast cancer cells differing in metastatic potential and in the number of EGFRs. SKBR-3 cells possess a high number of EGF, erbB2 and erbB3 receptors, while MCF7 cells possess a low number of EGFRs (Davidson *et al.*, 1987). Here, we have shown that as little as 5 ng/ml of EGF or 1 ng/ml of AR induce secretion of MMP-9 in SKBR-3 cells. The enzyme detected in the supernatant was in the latent form, as reflected by its molecular mass of 92 kDa. We detected up-regulation of the transcription factors AP-1[Fos/Jun] (data not shown), which are known to be stimulators of metalloproteinase genes (Sato and Seiki, 1993), but failed to detect mRNA levels of MMP-9 by Northern blot analysis. The effect of EGF and AR on MMP-9 secretion was specific for this enzyme since MMP-2 expression in SKBR-3 cells was not observed with these mitogens. This is consistent with the differential transcriptional regulation of MMP-9 and MMP-2 due to the presence of different promoter elements on the gelatinase genes (Brown *et al.*, 1990). The MMP-2 promoter has the characteristics of housekeeping genes and, as such, is constitutively expressed in many cultured cells. The promoter of MMP-9, like other members of the MMP family, contains, among other motifs, a TATA box and an AP1 element consistent with induction by a variety of cytokines, growth factors and oncogenes (Angel *et al.*, 1987).

Previous studies demonstrated stimulation of MMP-9 expression by EGF in keratinocytes (Lisa and Lurie, 1996) and colon carcinoma cells (Hyuga *et al.*, 1994). Thus, both normal and malignant cells produce MMP-9 in response to EGF. In our study, cell proliferation was significantly increased in response to EGF, AR and HRG in SKBR-3 and MCF-7 breast cancer cells. However, expression of MMP-9 in response to EGF and AR was induced in SKBR-3 but had no effect in MCF-7 cells. The reason for this difference is presently unknown. SKBR-3 and MCF-7 cells differ markedly in the number of EGFRs per cell. Thus, this difference may be related to the number of receptors. However, since EGF was able to induce MMP-9 in both normal and malignant keratinocytes (Lisa and Lurie, 1996), it is possible that the genetic background of each target cell may play a role in the differential effects of EGF and AR observed here. Nevertheless, these studies suggest that ligands inducing cell proliferation may also regulate other cellular activities associated with an invasive phenotype, including inducing enzymes involved in degradation of the extracellular matrix.

The proliferation or differentiation of breast cancer cells by EGF-like ligands is known to be mediated by type I receptors, including EGFR, erbB2, erbB3 and erbB4 depending on the cell lines (Peles and Yarden, 1993). Intriguingly, these presumably different pathways for growth and differentiation often use very similar intracellular signaling components in lower organisms (Doyle and Bishop, 1993). In SKBR-3 cells, interaction of HRG with erbB3 or erbB4 results in the transactivation of erbB2, with the concomitant stimulation of cell proliferation and lack of MMP-9 induction observed here. HRG, in contrast to EGF and AR, does not increase the autophosphorylation of EGFR. This suggests that MMP-9 induction may be predominantly mediated by EGFR-induced signal transduction.

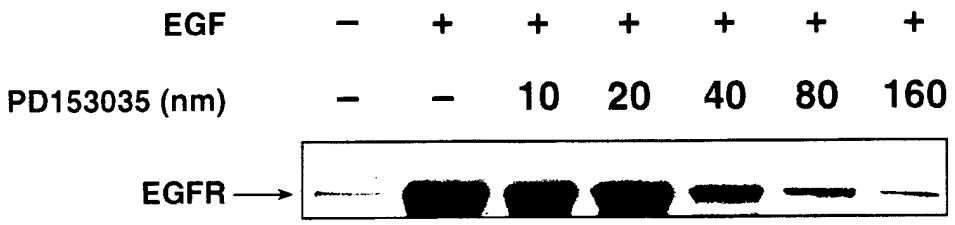

FIGURE 3 – Effect of PD 153035 concentration on autophosphorylation of EGFR in SKBR-3 cells. Cells were grown as shown in Figure 1, incubated for 2 hr with increasing concentrations of PD 153035, stimulated for 5 min with EGF (50 ng/ml) and lysed. EGFR phosphorylation was evaluated by Western blot analysis. Densitometric scanning of the autoradiogram suggests an LD_{50} of around 40 nM of PD 153035.

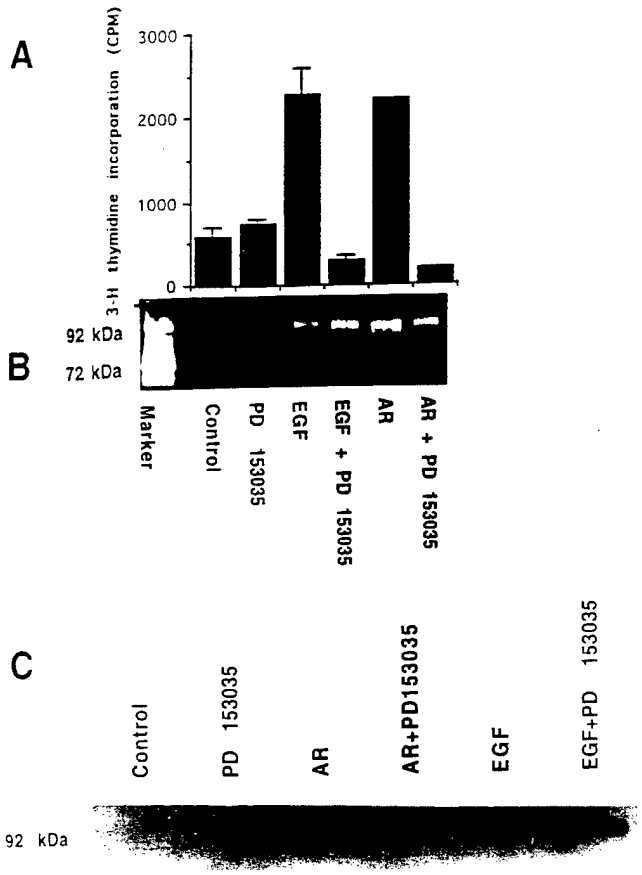

FIGURE 4 – Effect of PD 153035 on EGF- and AR-stimulated DNA synthesis and MMP-9 induction. SKBR-3 cells were grown in the presence of EGF and AR (50 ng/ml) with or without PD 153035. *(a)* [^{3}H]thymidine incorporation in SKBR-3 cells after 18 hr of incubation. Bars: mean ± SE of triplicate determinations. *(b)* MMP-9 induction by gelatin zymogram in the same condition as shown above. *(c)* Effects of PD 153035 on MMP-9 induction by Western blot analysis. PD 153035 significantly inhibited EGF- and AR-induced DNA synthesis but had no effect on the ability of EGF and AR to induce MMP-9.

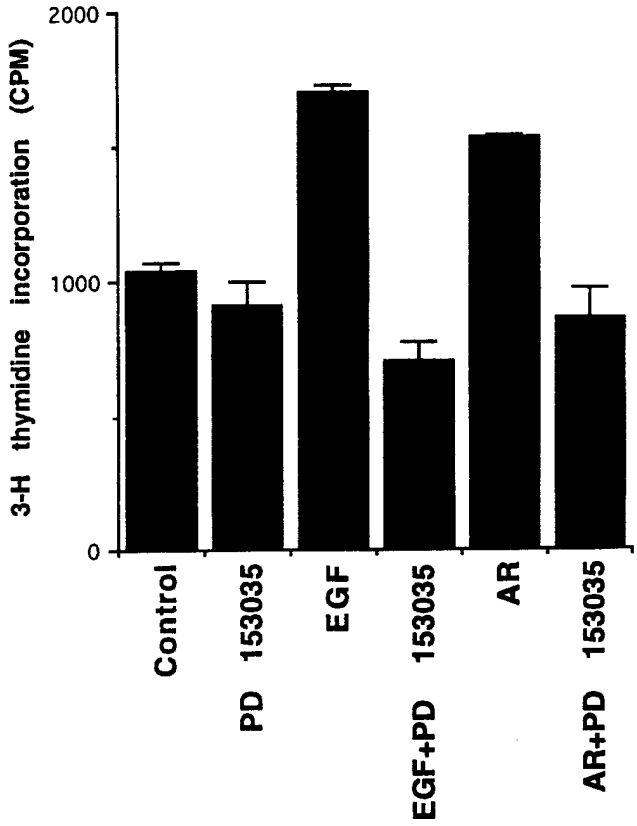

FIGURE 5 – Effect of PD 153035 on EGF- and AR-stimulated DNA synthesis. MCF-7 cells were grown in the presence of EGF and AR (50 ng/ml) with or without PD 153035. [^{3}H]thymidine incorporation in MCF-7 cells after 18 hr of incubation. Bars: mean ± SE of triplicate determinations. EGF and AR did not induce MMP-9 in MCF-7 cells.

To evaluate whether proliferation and MMP-9 were concurrently or independently acquired biological properties, we blocked cell proliferation with the specific EGF tyrosine kinase inhibitor PD 153035 (Fry *et al.*, 1994; Reddy *et al.*, 1992). PD 153035 was shown to be a relatively specific inhibitor of EGFR tyrosine kinase, and it suppresses autophosphorylation and selectively blocked EGF-mediated cellular processes including mitogenesis and early gene expression (Fry *et al.*, 1994). PD 153035 significantly inhibited EGF- and AR-induced cell proliferation (Fig. 4*a*) but had no effect on EGF- and AR-mediated induction of MMP-9 (Fig. 4*b*).

The inability of PD 153035 to block EGF- and AR-induced MMP-9 in spite of its ability to inhibit cell proliferation suggests that the signaling mechanism for cell proliferation and MMP-9 induction may be mediated by different pathways down-stream of EGFR autophosphorylation. Another possibility is that low levels of EGF- or AR-mediated signals that escape inhibition may be sufficient for MMP-9 induction but are unable to support cell proliferation.

In conclusion, we have shown that exposure of SKBR-3 cells to EGF (5 ng/ml) or AR (1 ng/ml) induces expression of MMP-9. This suggests that malignant cancers with high levels of EGFRs may induce MMPs in response to EGF present in serum (20–27 μM) (Hirata *et al.*, 1980) or tissue (1–5 ng/g tissue) (Hirata and Orth, 1979). Our results suggest that EGF and AR may modulate invasion of metastatic breast cancer cells by increasing the expression of MMPs.

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